

University of Dundee

## Structure-guided enhancement of selectivity of chemical probe inhibitors targeting bacterial seryl-tRNA synthetase

Cain, Ricky; Salimraj, Ramya; Puneekar, Avinash; Belini, Dom; Fishwick, Colin W. G.; Czaplewski, Lloyd

*Published in:*  
Journal of Medicinal Chemistry

*DOI:*  
[10.1021/acs.jmedchem.9b01131](https://doi.org/10.1021/acs.jmedchem.9b01131)

*Publication date:*  
2019

*Document Version*  
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

### *Citation for published version (APA):*

Cain, R., Salimraj, R., Puneekar, A., Belini, D., Fishwick, C. W. G., Czaplewski, L., Scott, D. J., Harris, G., Dowson, C. G., Lloyd, A. J., & Roper, D. I. (2019). Structure-guided enhancement of selectivity of chemical probe inhibitors targeting bacterial seryl-tRNA synthetase. *Journal of Medicinal Chemistry*, 62(21), 9703-9717. <https://doi.org/10.1021/acs.jmedchem.9b01131>

### General rights

Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Article

**Structure-guided enhancement of selectivity of chemical  
probe inhibitors targeting bacterial seryl-tRNA synthetase**

Ricky Cain, Ramya Salimraj, Avinash Puneekar, Dom Belini, Colin W. G. Fishwick, Lloyd Czaplewski,  
David Jan Scott, Gemma Harris, Christopher G Dowson, Adrian J. Lloyd, and David I. Roper

*J. Med. Chem.*, **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.9b01131 • Publication Date (Web): 18 Oct 2019

Downloaded from pubs.acs.org on November 4, 2019

**Just Accepted**

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

This document is the unedited author's version of a Submitted Work that was subsequently accepted for publication in *Journal of Medicinal Chemistry*, copyright © American Chemical Society after peer review. To access the final edited and published work, see <https://doi.org/10.1021/acs.jmedchem.9b01131>.

**Structure-guided enhancement of selectivity of chemical probe inhibitors  
targeting bacterial seryl-tRNA synthetase**

Ricky Cain,<sup>[a]†</sup> Ramya Salimraj,<sup>[a]†</sup> Avinash S. Punekar,<sup>[a]†</sup> Dom Bellini,<sup>[a]</sup> Colin W. G. Fishwick,<sup>[b]</sup>  
Lloyd Czaplewski,<sup>[c]</sup> David J. Scott,<sup>[d,e]</sup> Gemma Harris,<sup>[e]</sup> Christopher G. Dowson,<sup>[a]</sup> Adrian J. Lloyd,<sup>[a]</sup>  
David I. Roper<sup>[a]\*</sup>

<sup>[a]</sup> School of Life Sciences, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, United Kingdom.

<sup>[b]</sup> School of Chemistry, University of Leeds, Leeds, LS2 9JT, United Kingdom.

<sup>[c]</sup> Chemical Biology Ventures Limited, Abingdon, OX14 1XD, United Kingdom

<sup>[d]</sup> School of Biosciences, University of Nottingham, Nottingham, LE12 5RD, United Kingdom

<sup>[e]</sup> ISIS Spallation Neutron and Muon Source and the Research Complex at Harwell, Rutherford Appleton Laboratory, Oxfordshire OX11 0FA, United Kingdom

<sup>†</sup> These authors contributed equally to this work

<sup>†</sup>Present address – The Institute of Cancer Research, 15 Cotswold Road, Sutton, SM2 5NG, United Kingdom

<sup>††</sup> Present address - Drug Discovery Unit, School of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom.

\* E-mail: [david.roper@warwick.ac.uk](mailto:david.roper@warwick.ac.uk)

## Abstract

Aminoacyl-tRNA synthetases are ubiquitous and essential enzymes for protein synthesis and also a variety of other metabolic processes, especially in bacterial species. Bacterial aminoacyl-tRNA synthetases represent attractive and validated targets for antimicrobial drug discovery if issues of prokaryotic versus eukaryotic selectivity and antibiotic resistance generation can be addressed. We have determined high resolution X-ray crystal structures of the *Escherichia coli* and *Staphylococcus aureus* seryl-tRNA synthetases in complex with aminoacyl adenylate analogues and applied a structure-based drug discovery approach to explore and identify a series of small molecule inhibitors that selectively inhibit bacterial seryl-tRNA synthetases with greater than two orders of magnitude compared to their human homologue, demonstrating a route to selective chemical inhibition of these bacterial targets.

## Introduction

The fidelity of protein synthesis is absolutely reliant upon the provision of specific amino acids by aminoacyl-tRNA molecules for use by the ribosome.<sup>1</sup> Errors in this process cause defects in protein folding and function leading to cell death.<sup>2</sup> Each of the 20 amino acids has its own aminoacyl-tRNA synthetase (aaRS) which catalyses the attachment of the amino acid to its cognate tRNA. Despite the fact that all aaRSs share the same overall mechanism, it has long been recognised that there is clearly significant diversity between bacterial, mammalian and archaeal enzymes to allow for synthetic and natural product discrimination between pathogen and host enzymes<sup>3-5</sup>. In addition, in some situations, several different amino acids are able to bind to non-cognate aaRSs, requiring an *in vivo* editing function allowing for the possibility of exploiting this feature for future antimicrobial discovery<sup>6</sup>. For example, the amino acid serine is able to bind alanyl-tRNA synthetase (AlaRS) and threonyl-tRNA synthetase (ThrRS) in addition to its cognate seryl-tRNA synthetase (SerRS)<sup>7</sup>. This incorrect binding is rectified in nature by numerous proofreading mechanisms<sup>6, 8</sup>. However, in this context, one of the major challenges presented by aaRS as targets for antimicrobial drug discovery is their ubiquitous presence in organisms and particularly with respect to bacterial infection in human tissues requiring exploration of strategies that allow for bacterial selectivity to prevent issues of specificity and toxicity<sup>9</sup>.

Aminoacyl sulfamoyl adenosines (aaSAs) are non-hydrolysable mimetics of the aminoacyl adenylate intermediate (aaAMP) formed during the aaRS catalytic cycle and are potent inhibitors of these enzymes.<sup>10</sup> A significant number of natural product inhibitors mimic these reaction intermediates forming tight binding complexes with substantial affinity competing effectively with natural aaAMP substrates. Of those, mupirocin is the most prominent example that has found clinical utility as a topical treatment for soft tissue infections. Mupirocin targets the IleRS enzyme and utilises a hydrophobic “tail” in addition to an aminoacyl

adenylate warhead to bind to its target<sup>11</sup>. By contrast to many single target antibiotics in clinical use, seryl sulfamoyl adenosine (SerSA, **1**) can bind and inhibit AlaRS and ThrRS in addition to SerRS and hence is a multi-targeting inhibitor<sup>7, 12</sup>. It can be predicted therefore that SerSA would require mutations in several of these enzymes before a resistance phenotype could be conferred.

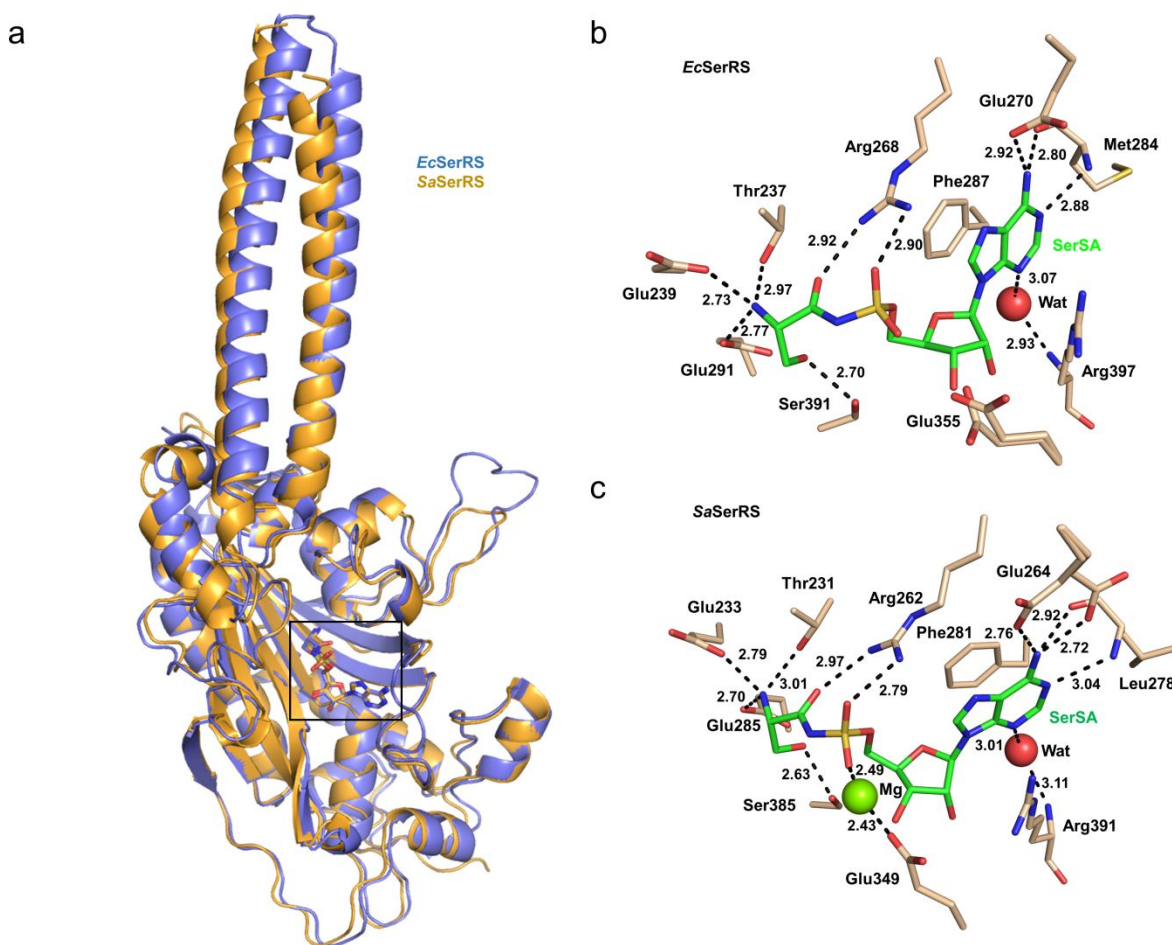
The protein databank (PDB) contains X-ray crystal structures of SerRS from *Thermus thermophilus*<sup>13, 14</sup>, *Methanosarcina barkeri*<sup>15</sup>, *Pyrococcus horikoshii*<sup>16</sup>, *Candida albicans*<sup>17</sup>, *Arabidopsis thaliana*<sup>18</sup>, *Methanopyrus kandleri*<sup>19</sup>, *Trypanosoma brucei*, human cytoplasmic<sup>20</sup> and bovine mitochondrial<sup>21</sup>. It is therefore evident that there is a distinct lack of structural data available for clinically relevant bacteria. Although the *Escherichia coli* SerRS<sup>22</sup> structure was solved in 1990, the coordinates were not deposited in the PDB thereby hampering efforts in antimicrobial structure-based drug discovery (SBDD) based on this structure. Moreover, the X-ray crystal structure of human SerRS in complex with SerSA reveals specific conformational changes upon catalysis necessary for function, which are not found in bacterial homologues providing further perspectives upon differences in structure that may allow prokaryotic from eukaryotic specificity.<sup>20</sup> In this study we set out to increase the available structural information for human bacterial pathogens and use this to investigate the possibilities for designing bacteria-specific SerRS enzyme inhibitors using a SBDD approach.

## Results & Discussion

### Crystal structures of SerRS in complex with SerSA inhibitor.

The crystal structures of full-length SerRS from *E. coli* (*EcSerRS*) and *Staphylococcus aureus* (*SaSerRS*) in complex with the SerSA inhibitor were solved at 1.50 Å and 2.03 Å, respectively (**Fig. 1, Supplementary Table 1**). In both structures, the SerSA inhibitor is unambiguously determined by the electron density maps (**Supplementary Fig. 1**). SerSA is bound deep into a well-conserved SerRS aminoacylation catalytic pocket and stabilized by a network of hydrogen bond interactions from the residues in motif 2, motif 3 and the serine-binding TxE motif (**Fig. 1a**) - a typical binding mode in all class 2 aaRSs. Superimposition of *EcSerRS*, *SaSerRS* and human cytoplasmic SerRS (*HsSerRS*, PDB ID: 4L87<sup>20</sup>) structures show a high degree of similarity as evidenced by the RMSD values (**Supplementary Table 2**). The orientation of the bound SerSA inhibitor is comparable in all three structures. However, the N-terminal tRNA-binding domain (i.e. the two-stranded anti-parallel coiled coil making the long helical arm) protruding away from the active site pockets in the compared structures shows large conformational changes resulting in a high RMSD.<sup>20</sup> The purine ring of the adenosine in SerSA interacts with a conserved phenylalanine (F287 in *EcSerRS*, F281 in *SaSerRS* and F321 in *HsSerRS*) via a  $\pi$ - $\pi$  stacking interaction (**Fig. 1b**). The M284 in *EcSerRS*,

L278 in *SaSerRS* and V318 in *HsSerRS* are positioned such that they provide main chain hydrogen bond interactions with the ring nitrogens (**Fig. 1c**). The seryl moiety of SerSA extends deep into the pocket to interact with T237, E239, R268, E291 and S391 in *EcSerRS* and equivalent residues in *SaSerRS* and *HsSerRS*. We note the presence of a highly coordinated water molecule 3 Å away from the N<sup>3</sup> of the adenine moiety of the adenylyate (**Fig. 1b-c**), a feature that has previously been described in class II synthetase enzymes.<sup>23</sup> In *SaSerRS* the octahedral coordination of a magnesium ion (**Fig 1c**) is observed in the active site via Glu349, the SerSA sulfone and water molecules, reminiscent of the magnesium ion observed in both the *Candida albicans* SerRS and *HsSerRS*.



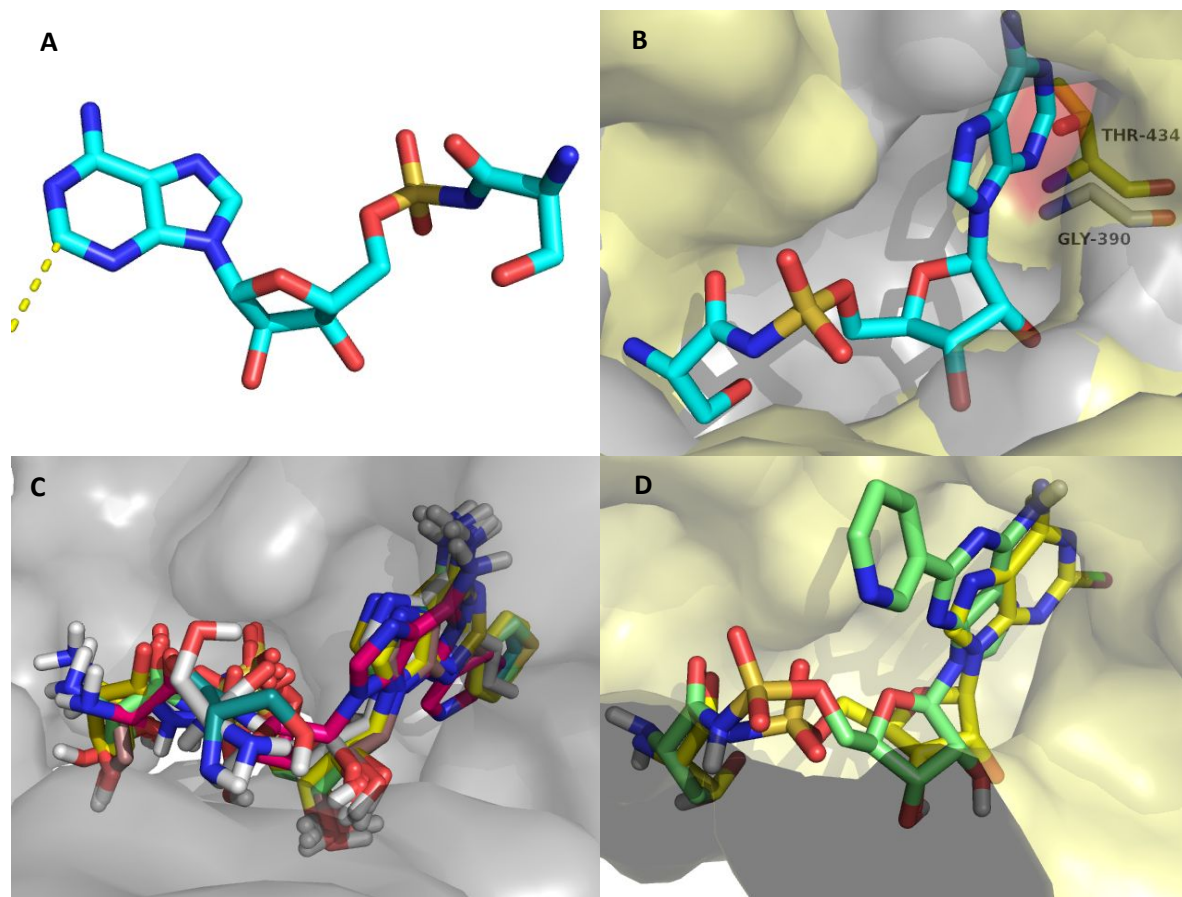
**Figure 1: Binding mode of SerSA to *E. coli* and *S. aureus* SerRS.** a: Superposition of *EcSerRS* (blue, PDB ID: 6R1M) and *SaSerRS* (gold, PDB ID: 6R1N) with SerSA bound (boxed). b: Interactions of SerSA (green sticks) with *EcSerRS* chain A. Water represented as a red sphere. Hydrogen bond interactions shown as black dashes. c: Interactions of SerSA with *SaSerRS*. Coordinated magnesium ion represented as a green sphere.

### Design and synthesis of the selectivity probe.

The X-ray crystal structures of *Ec*SerRS, *Sa*SerRS and the *Hs*SerRS (PDB ID: 4L87) were superimposed in Maestro (Schrödinger, LLC).<sup>24</sup> Interestingly, a thorough analysis of the active site pockets revealed a small extension in the hydrophobic cavity adjacent to the C-2 position of SerSA (**1**) in the *Ec*SerRS and *Sa*SerRS structures. This pocket is centred around a glycine at positions 396 and 390 in *Ec*SerRS, *Sa*SerRS respectively. This hydrophobic cavity extension is absent in the *Hs*SerRS (**Fig. 2b**) as it is filled by the bulkier side-chain of threonine at position 434. The conserved nature of this structural difference is reflected in amino acid sequence alignments of selected Gram-positive and Gram-negative bacterial pathogens when compared to cytoplasmic and mitochondrial variants of the human, bovine and mouse SerRS enzymes (**Supplementary Fig. 2a**). Moreover, inspection of an alignment of the Gram-positive and Gram-negative bacterial pathogens shows this glycine to be part of a 12 amino acid region of conservation ending in an arginine (397 in *Ec*SerRS, 391 in *Sa*SerRS) suggestive of an invariant bacterial structural feature absent in eukaryotic homologues. (**Supplementary Fig. 2b**). Exploiting such a conserved feature for antimicrobial drug discovery extends the range of bacteria that can potentially be targeted whilst also reducing the chances of mutation-induced drug resistance.

A focused structure-activity relationship (SAR) series with variants at the C-2 position of SerSA adenosine was designed to investigate the steric tolerance of the hydrophobic cavity and to establish the degree of selectivity for the bacterial SerRS over the *Hs*SerRS (**Fig. 3a**). *In silico* molecular docking of the designed selectivity probes into the active site pockets of the *Ec*SerRS, *Sa*SerRS and *Hs*SerRS crystal structures (**Supplementary Methods, Supplementary Table 3**) and visual analysis of the predicted docking poses (**Fig. 2c-d**) suggested that chloro- and iodo-seryl sulfamoyl adenylate derivatives **2** and **3** respectively would not achieve selectivity since **2** and **3** were predicted to interact equally as well with both the bacterial and *Hs*SerRS. Compounds **4-8** were however predicted to exhibit selectivity for the bacterial SerRS over the *Hs*SerRS.





**Figure 2: Binding modes of designed seryl sulfamoyl adenylate selectivity probes.** (a) 3D spatial representation of the seryl sulfamoyl adenylate derivatives indicating C-2 position where SAR study was focussed (yellow dashed line). (b) Structural overlay of *SaSerRS* (Grey, PDB ID 6R1N) and *HsSerRS* (Yellow, PDB ID: 4L87) active site showing the key residue change near the 2 position of the sulfamoyl adenylate inhibitor from Gly390 in the bacterial form to Thr434 in the human form. (c) Predicted binding modes of seryl sulfamoyl adenylates to *SaSerRS* (PDB ID 6R1N) using AutoDock 4.2. (d) Predicted binding modes of seryl sulfamoyl adenylates to *HsSerRS* using AutoDock 4.2.

The bulkier groups located at the 2 position of compounds **4-8** were predicted to be accommodated in the pocket of the bacterial enzymes. However, due to the steric hindrance from the T434 residue in the *HsSerRS*, compounds **4-8** were predicted to change the torsional angle between the adenine and ribose sugar upon binding to the *HsSerRS*. As a result of the torsional change the  $\pi$ - $\pi$  stacking interactions with F287 and the backbone interaction to V318 are lost leading to a weaker predicted binding affinity and therefore increased selectivity for the bacterial SerRS (**Fig. 2d**).

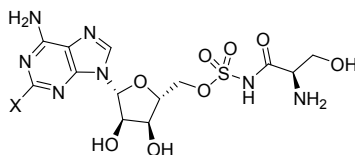


Preparation of SerSA selectivity probes was initiated by the acid-catalysed protection of the commercially available 2-chloroadenosine or 2-iodoadenosine (Fluorochem, UK) to provide the acetyl-protected adenosines (95-97%) (**Scheme 1, 9-10**). A Suzuki coupling reaction between the protected adenosine and desired boronic acid species (20-70%) was conducted (**11-15**),<sup>25</sup> before sulfonation using sulfonyl chloride to afford the sulfonamide (90-95%) (**16-22**). The sulfonamide was then coupled to the succinimide activated protected serine (**23**) to yield the protected product (40-50%) (**24-30**). Removal of the benzyl group was accomplished by treatment with a solution of boron trichloride dimethyl sulfide complex (2M in DCM),<sup>26</sup> and the resulting alcohol was treated with trifluoroacetic acid and water to yield compounds **2-8** (**2-8**, see **Experimental Section** and **Supplementary Information** for details).

### Bacterial SerRS inhibition by selectivity probe

Using a continuous spectrophotometric assay that specifically measures the adenylate formation reaction<sup>7</sup>, compounds **2-8** were evaluated for the inhibition of ATP-dependent aminoacyl adenylate formation by *Ec*SerRS and *Sa*SerRS enzymes and compared their half maximal inhibitory concentration (IC<sub>50</sub>) values with the parent SerSA, compound **1** (**Supplementary Table 4 and 5**). Compounds **2-8** were active against *Ec*SerRS and *Sa*SerRS with IC<sub>50</sub> values ranging from 378 nM to 52.7 μM (**Table 1**). Compound **2** exhibited sub-micromolar inhibition of the *Sa*SerRS and *Ec*SerRS with IC<sub>50</sub>s of 262 nM and 445 nM respectively. Compound **3** also exhibited sub-micromolar inhibition of *Sa*SerRS with an IC<sub>50</sub> of 378 nM but weaker inhibition of *Ec*SerRS with an IC<sub>50</sub> of 1.36 μM. Compounds **4-8** all manifested low micromolar inhibition against both bacterial SerRS (**Table 1**). A general trend is observed where increasing the size of the group at the 2 position of the adenylate decreases the binding affinity to the bacterial synthetase. Alanyl sulfamoyl adenosine (AlaSA, **31**) and threonyl sulfamoyl adenosine (ThrSA **32**) were also evaluated for inhibition against *Ec*SerRS and *Sa*SerRS (**Supplementary Table 6**). AlaSA **31** showed no inhibitory activity against either enzyme at 1 mM while ThrSA **32** manifested IC<sub>50</sub>s of 285 μM and 231 μM against *Ec*SerRS and *Sa*SerRS respectively, thus exhibiting much weaker binding than the designed selectivity probes. These results highlight the key role of the beta-hydroxyl of the serine to the overall binding of the compound within the adenylate formation site in these enzymes and the overall inhibitory properties of seryl adenylate inhibitors modified around the C-2 position of the SerSA adenosine.

**Table 1: IC<sub>50</sub> values of designed chemical probes against seryl-tRNA synthetases. Assays were conducted as reported.<sup>7</sup>**



No.	X	IC <sub>50</sub> <i>EcSerRS</i> (μM)	IC <sub>50</sub> <i>SaSerRS</i> (μM)	IC <sub>50</sub> <i>HsSerRS</i> (μM)
<b>1 (SerSA)</b>	H	0.21 ± 0.03	0.23 ± 0.49	2.17 ± 0.21
<b>2</b>	Cl	0.45 ± 0.05	0.26 ± 0.03	67.3 ± 4.67
<b>3</b>	I	1.36 ± 0.12	0.38 ± 0.04	24.0 ± 2.26
<b>4</b>	C <sub>6</sub> H <sub>5</sub>	17.7 ± 1.42	52.7 ± 4.81	>1000 ± >100
<b>5</b>	<i>trans</i> -Propenyl	9.38 ± 0.70	3.46 ± 0.47	>1000 ± >100
<b>6</b>	2-Furyl	36.2 ± 2.41	32.4 ± 3.56	>1000 ± >100
<b>7</b>	3-Thienyl	1.44 ± 0.09	1.24 ± 0.12	>1000 ± >100 (ppt)
<b>8</b>	3-Pyridyl	6.65 ± 0.64	6.34 ± 0.71	>1000 ± >100 (ppt)

SerRS, Seryl t-RNA synthetase. (ppt) precipitation observed at 1000 μM.  
Errors were calculated as s.d. of at least three independent measurements.

### ***HsSerRS* inhibition by selectivity probes**

Measurement of the IC<sub>50</sub> inhibition kinetics of the original SerSA, compound **1** against the bacterial and human SerRS enzymes, reveals a 10-fold difference, in favour of greater specificity of the inhibitor for the bacterial enzymes. Compounds **2-8** were subsequently screened for inhibition of the *HsSerRS* (**Table 1**) using the same assay system. Assay measurements of compounds **2** and **3**, revealed a 31-fold and 11-fold increase in IC<sub>50</sub> against the bacterial SerRS and *HsSerRS*, indicating that compounds **2** and **3** did not exhibit selectivity overall and had lower affinity than SerSA **1**. Overall the observed IC<sub>50</sub> of compounds **2-8** increased with respect to the parental adenylyate **1** but remarkably, inhibition of the *HsSerRS* was effectively abolished in compounds **4-8** with IC<sub>50</sub> values greater than 1 mM, revealing significant selectivity of these compounds towards the tested bacterial SerRS. The best of these compounds (**7**), with a 3-Thienyl at the C-2 position of the SerSA adenosine had an increase in IC<sub>50</sub> over SerSA **1** of 6.8 and 8.4 fold for *EcSerRS* and *SaSerRS* respectively, with effectively negligible binding to the *HsSerRS*. The observed selectivity overall was attributed to the increased size of **4-8** making them unable to fit into the hydrophobic pocket located in the human cytoplasmic SerRS active site due to the presence of T434 as previously hypothesised.

### **Binding studies of SerSA and compound **8** to *EcSerRS***

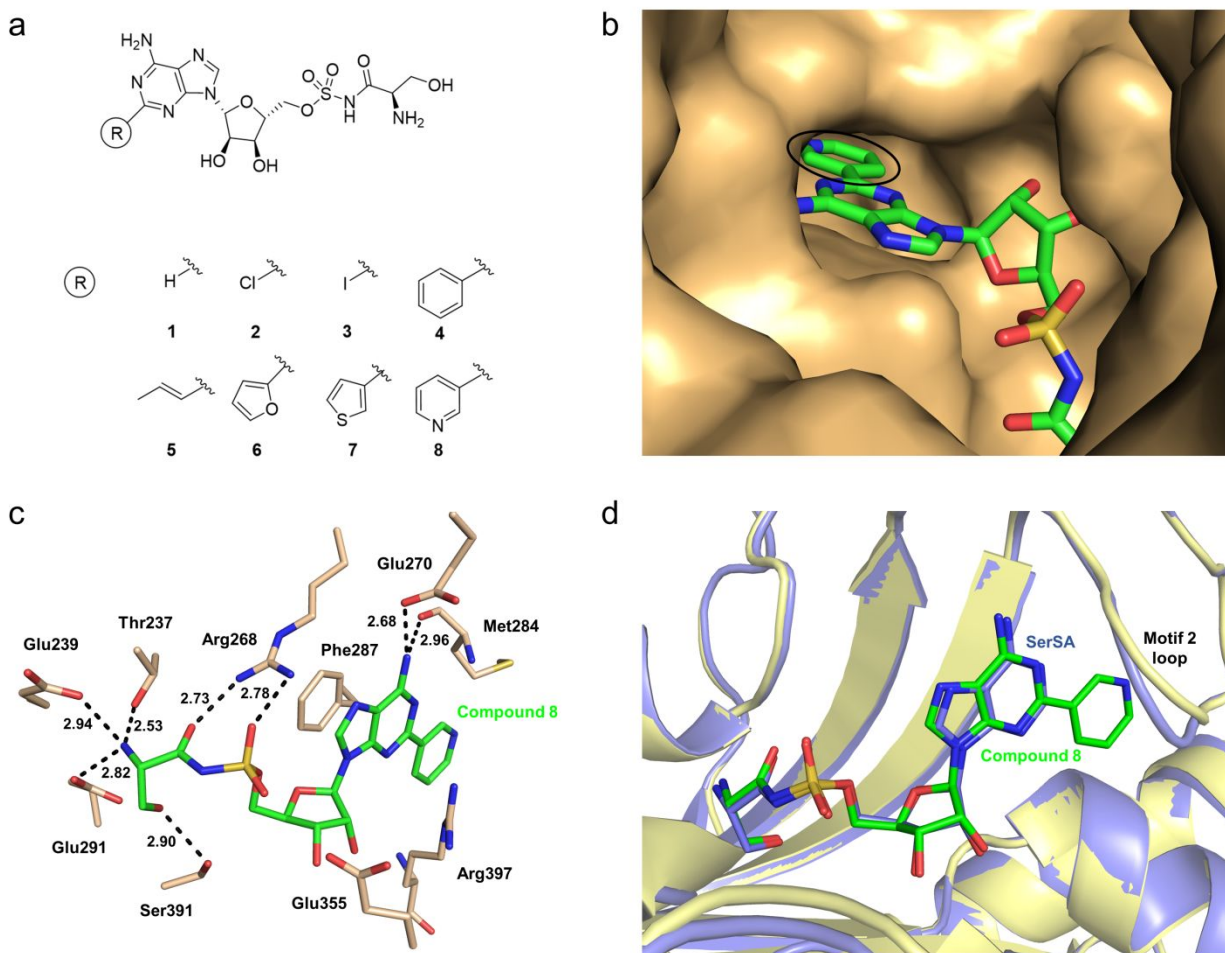
To independently measure the binding characteristics of the original adenylyate SerSA and the derivatives synthesised in this study, we measured binding affinity using iso isothermal titration calorimetry (ITC). The binding stoichiometry and affinity of SerSA **1** and compound **8** to *EcSerRS* was determined using ITC because compound **8** had the best solubility of the synthesised compounds. Titration of SerSA to *EcSerRS* resulted in a steep slope in the binding isotherm suggesting a very tight binding of the inhibitor to the

enzyme. Interestingly, fitting of this binding isotherm using a single site model showed a 2:1 SerSA:SerRS stoichiometry with an overall dissociation constant  $K_d = 1.27$  nM (**Supplementary Fig. 3a**). The combination of very high affinity and low enthalpy unfortunately prevented an accurate measurement of  $K_d$  for SerSA at the individual binding sites.

By contrast, titration of compound **8** to *Ec*SerRS resulted in a binding isotherm (2:1 compound **8**:SerRS stoichiometry) that after fitting using a two independent sites model clearly showed two distinct binding sites with dissociation constants  $K_{d1} = 0.29$   $\mu$ M and  $K_{d2} = 1.92$   $\mu$ M (**Supplementary Fig. 3b**). As  $K_{d2} > 4 K_{d1}$ , there is apparent mild negative cooperativity within the system. In both experiments, a negative enthalpy value detected for such a tight interaction indicates the role of hydrogen bond and electrostatic interactions in the stabilisation of the enzyme–inhibitor complex. The observation of two binding sites for SerSA and compound **8** prompted us to investigate the oligomeric state of the *Ec*SerRS in solution, which are typically dimers in solution.<sup>27</sup> Analytical ultracentrifugation (AUC) experiments were carried out with *Ec*SerRS to confirm the oligomeric state of the protein in the presence and absence of SerSA and compound **8** (**Supplementary Table 6**). The results confirmed that *Ec*SerRS, both with and without inhibitors, appeared with a molecular weight that is consistent with a dimer in solution (**Supplementary Fig. 4**). The observed SerSA and compound **8** binding stoichiometry is consistent with the previous structural findings showing two SerSA molecules bound to two distinct sites in the X-ray crystal structure of *Candida albicans* SerRS (PDB ID: 3QO8)<sup>17</sup>. In this structure the second SerSA binding site is located 26 Å distant from the active site and appears to play no role in the enzyme function or protein-protein interaction as described by the authors<sup>17</sup>.

### Structural basis of selectivity probe binding to *Ec*SerRS

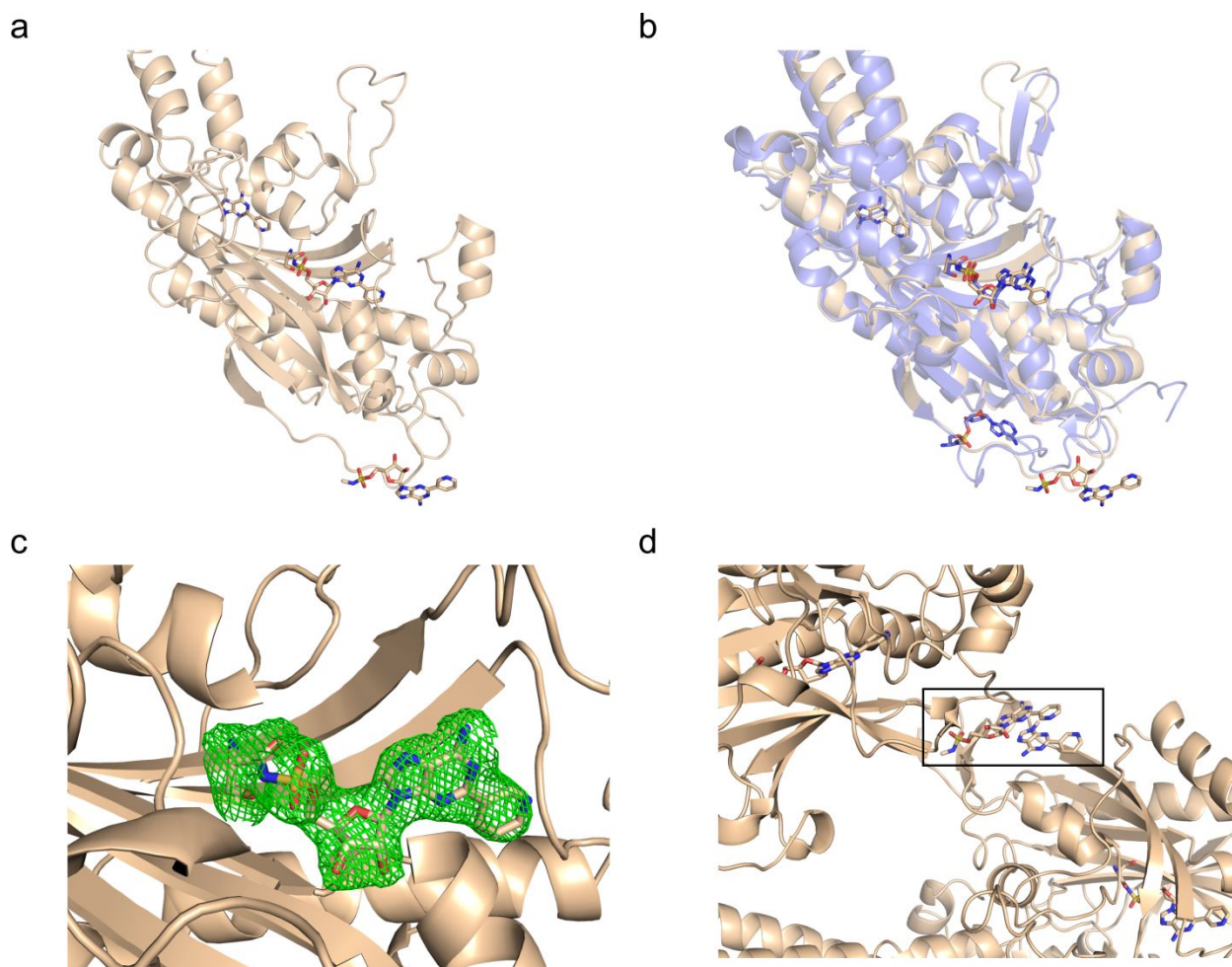
To understand the molecular basis of the selectivity probe towards bacterial SerRS, we attempted a series of co-crystallisation screening of *Ec*SerRS and *Sa*SerRS. Despite extensive screening, we were unable to find hit conditions to co-crystallise *Sa*SerRS in the presence of compound **7** or **8**. However, we were successful in obtaining crystals of *Ec*SerRS amenable to soaking with compound **8**, which yielded a 2.6 Å resolution structure (**Fig. 3a-d**). The *Ec*SerRS-SerSA complex structure was solved in the space group P1 containing two monomers that associate tightly to form a dimer. In contrast, the *Ec*SerRS-compound **8** complex structure was solved in space group P6<sub>1</sub>22 with 1 molecule in the asymmetric unit. Compound **8** binds in a similar fashion to SerSA in *Ec*SerRS making key interactions with the residues in motif 2, motif 3 and the serine-binding TxE motif as described above (**Fig. 3c**). The 3-pyridyl group of compound **8** snugly fits into the hydrophobic cavity without any other obvious interactions (**Fig. 3b**) with movement of the motif 2 loop observed to accommodate the pyridyl group (**Fig. 3d**).



**Figure 3: Comparison of binding of SerSA and compound 8 to *EcSerRS* active site.** **a:** The chemical structures of the compounds used in this study. **b:** Pyridyl group of compound 8 (circled) positioned in the active site. **c:** Interactions of compound 8 (green sticks) with *EcSerRS*. Hydrogen bond interactions are shown as black dashes. **d:** Superposition of *EcSerRS*:SerSA (blue, PDB ID:6R1M) with *EcSerRS*:compound 8 (PDB ID:6R1O).

We analysed both structures for presence of a second adenylylate-binding site as found in the *Candida albicans* SerRS-SerSA structure (PDB ID: 3QO8)<sup>17</sup>. No density for the second adenylylate was found in the *EcSerRS*-SerSA structure, but density for two additional ligand molecules were observed in the *EcSerRS*-compound 8 structure (**Fig. 4a**). These ligands were found to bind away from the active site in positions distinct to that observed in the *Candida albicans* SerSA (**Fig. 4b**). Electron density for the complete compound was observed for the ligand in the active site (**Fig. 4c**) and a second ligand which  $\pi$ - $\pi$ -stacks with a third ligand from a symmetry-related molecule for which electron density is only observed for its purine and pyridyl rings (**Fig. 4d**). The seryl moiety of this third ligand is likely to not make any interactions with the protein and be flexible due to the absence of electron density for this region of the compound. As

such the presence of this third ligand molecule is likely to be a crystallographic artefact of the high concentration of compound used for soaking and this structure provides evidence for a potential second binding site that is supported by the ITC data.



**Figure 4: Second binding site of compound 8 to *EcSerRS*.** a: Binding positions of compound 8 to *EcSerRS*. b: Overlay of *EcSerRS*:compound 8 (wheat, PDB ID: 6R1O) with *Candida albicans* SerRS (blue, PDB ID: 3QO8) c: Overlay of  $F_o - F_c$  omit map of compound 8 in *EcSerRS* active site contoured at  $\sigma$  3. d: Interaction of two molecules of compound 8 (boxed) from *EcSerRS* symmetry-related molecules.

#### Pathogen susceptibility testing to selectivity probes.

Previous studies by Van de Vijver *et. al.* showed that SerSA (**1**) did not exhibit an MIC against *S. aureus* and *E. coli* in disk diffusion studies<sup>10</sup>. Compounds **1-8** were screened in an antimicrobial susceptibility assay using CLSI guidelines<sup>28</sup> to determine MIC against both *S. aureus* and *E. coli* however no MIC's were observed (>256 ug/ml). This result is consistent with the previous reported studied of **1** and is likely to be the result of poor cell permeability or efflux after the molecules enter the cell. In order to rationalise the poor activity against bacterial cells the compounds produced in this study were analysed using the bioinformatics tool Entryway ([www.entry-way.org](http://www.entry-way.org)), which classifies molecules that are likely to be capable

of accumulating in Gram-negative bacteria. Whilst the compounds fulfil the requirements for globularity and contain the required primary amine the number of rotatable bonds exceeds the limits normally founds in antimicrobials.<sup>29</sup> The compounds described would therefore require further lead optimisation to progress them from selective inhibitors to the final desired antimicrobials.

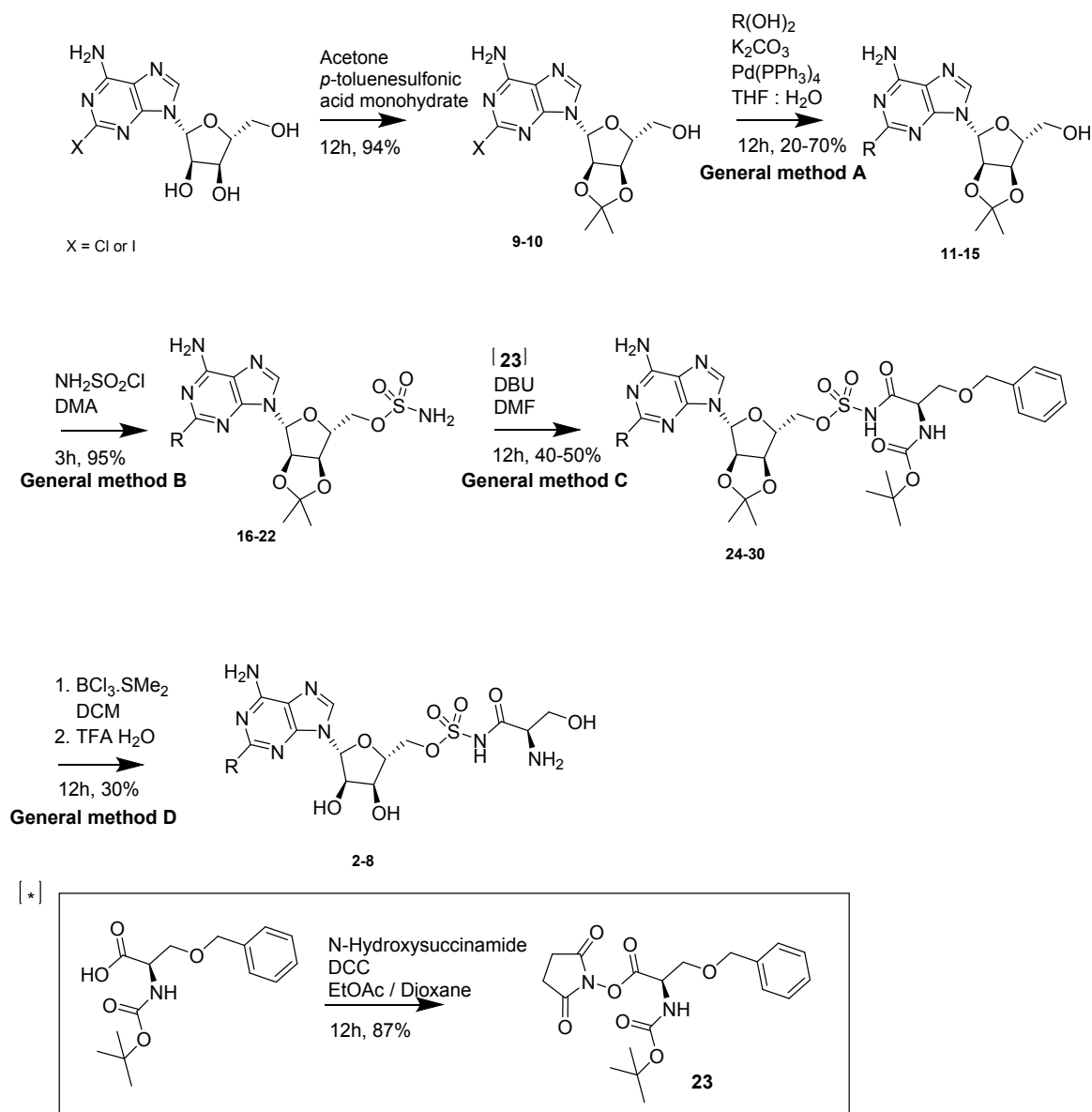
## Conclusion

In summary, we demonstrate the use of structure-based drug design to identify selective inhibitors of exemplar SerRS enzymes from Gram-positive and Gram-negative pathogens on the WHO list of bacteria for which new antibiotics are urgently needed. Previous studies have investigated inhibiting protein synthesis via inhibition of specific aaRS activities leading to the identification of a number of potent antibiotics which have progressed through into clinical studies<sup>17, 30-33</sup>. Rapid development of resistance to these synthetase inhibitors has halted their clinical evaluation<sup>34</sup>. The reported alternative approach herein has been a proof of principle example of the capability of structure-based drug design in modifying a multi-targeting aaRS inhibitor to achieve selectivity.

Analysis using the bioinformatics tool Entryway ([www.entry-way.org](http://www.entry-way.org)), showed that whilst the compounds fulfil the requirements for globularity and contain the required primary amine the number of rotatable bonds exceeds the limits normally founds in antimicrobials.<sup>29</sup> Further work is required to achieve clinically viable compounds that can permeate the cell membrane but the crystal structures here, nonetheless, provide a foundation for structure-based drug design of novel selective inhibitors which multi-target the bacterial aminoacyl-tRNA synthetases.

## Experimental

### Scheme1: Synthetic route of target SerSA selectivity probes



**General information in synthetic chemistry.** Chemicals were from commonly used suppliers and used without further purification. SerSA (**1**), AlaSA (**31**) and ThrSA (**32**) were purchased from Sythesis MedChem (UK) Ltd (Cambridge, UK). Solvents (including dry solvents) for chemical transformations, work-up and chromatography were from Sigma-Aldrich (Dorset, UK) at HPLC grade, and used without further distillation. Silica gel 60 F254 analytical thin layer chromatography (TLC) plates were from Merck (Darmstadt, Germany) and visualized under UV light and/or with potassium permanganate stain. Chromatographic purifications were performed using Merck Geduran 60 silica (40-63  $\mu\text{m}$ ) or prepacked SNAP columns on a Biotage Isolera Purification system (Uppsala, Sweden). Deuterated solvents were from Sigma-Aldrich, Chambridge Isotopes and Apollo Scientific Ltd. All  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded using a Bruker Avance 500 MHz or Bruker Avance 400 MHz spectrometer. All chemical shifts are in ppm



relative to the solvent peak, and signal splitting patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), quintet (quint), or multiplet (m) with coupling constants (J) are reported in Hz to the nearest 0.5. High Resolution (HR) mass spectrometry data (m/z) were obtained using a Bruker MaXis Impact instrument with an ESI source and Time of Flight (TOF) analyzer. Fourier transform Infrared (FT-IR) spectra were recorded on a Bruker Alpha Platinum instrument. Melting points were obtained from a Reichert Hot Stage melting point apparatus. HPLC analysis was run on an Agilent 1290 Infinity system equipped with a Supelco Ascentis Express 2.7  $\mu$ M C18 column (50 x 2.1 mm) using a gradient of 95% solvent A  $\rightarrow$  95% solvent B (solvent A: H<sub>2</sub>O containing 0.1% formic acid; solvent B: 100% MeCN containing 0.1% formic acid), flow rate = 0.5 mL/min and UV detection at 254 nm. Specific rotation measurements were recorded using a Schmidt and Haensch Polartronic H532 polarimeter, using a 100 mm cell and the Sodium D line (589 nm).  $[\alpha]_D$  are reported in units of 10<sup>-1</sup> deg dm<sup>2</sup>g<sup>-1</sup>. The purities of all of the final compounds for biological testing were determined to be over 95% by NMR and HPLC. See the Supporting Information for <sup>1</sup>H and <sup>13</sup>C NMR spectra, HR Mass spectrometry and HPLC purity analysis of all compounds.

**General method A: Suzuki coupling reactions (11-15).** The boronic acid (4.0 equiv.) was added to a stirred solution of 2-chloroadenosine (1.0 equiv.), potassium carbonate (2.00 equiv.) and tetrakis (triphenylphosphine) palladium (0) (0.20 equiv.) in THF (8 mL) and water (4 mL). The reaction mixture was heated to reflux for 12 h. The reaction mixture was filtered through Celite® and concentrated *in vacuo*. The residue was diluted with water (20 mL) and extracted in EtOAc (3  $\times$  20 mL). The combined organics were washed with water (3  $\times$  10 mL) and brine (3  $\times$  10 mL), dried (MgSO<sub>4</sub>) and concentrated *in vacuo* to give an off-white/yellow solid, which was purified using flash column chromatography to afford the coupled products which were used without further purification.

**General method B: Introduction of sulfonyl group (16-22).** Generation of sulfanoyl chloride: Chlorosulfonyl isocyanate (1 mL, 11.5 mmol, 1.0 equiv.) was cooled to 0 °C under an atmosphere of nitrogen. Formic acid (0.43 mL, 11.5 mmol, 1.0 equiv.) was added dropwise and the mixture stirred at room temperature overnight. Gas evolution was observed. The resulting colourless solid was dried *in vacuo*. The colourless solid was used without further purification / characterisation. Sulfanoyl chloride (2.0 equiv.) in DMA (2 mL) was added dropwise to a stirred mixture of acetyl protected adenolate (1.0 equiv.) in DMA (3 mL) at 0 °C under an atmosphere of nitrogen. The mixture was then stirred at room temperature for 3 h. The reaction mixture was quenched with Et<sub>3</sub>N (1.5 mL) then MeOH (5 mL). The resulting solution was concentrated *in vacuo* before EtOAc (50 mL) was added. The mixture was extracted with 5% NaHCO<sub>3</sub> (3

× 15 mL), brine (3 × 10 mL), dried (MgSO<sub>4</sub>) and concentrated *in vacuo* to afford the desired product as a colourless glassy solid.

**General method C: Amide coupling reaction (24-30).** Sulfonamide adenylate (1.0 equiv.) was dissolved in DMF (10 mL). After addition of DBU (1.1 equiv.), N-Boc-Ser(bzl)-OSu (**23**) (1.1 equiv.) was added to the reaction mixture. After stirring for 16 h at room temperature, the mixture was concentrated *in vacuo* and the residue taken up in water (50 mL) and extracted with dichloromethane (50 mL). The organic layers were dried (MgSO<sub>4</sub>) concentrated *in vacuo* and purified by flash chromatography (EtOAc) to afford the desired product as a colourless powder.

**General method D: Global deprotection (2-8).** Seryl-sulfonamide adenylate (1.0 equiv.) was dissolved in DCM (5 mL) under an atmosphere of nitrogen. To this was added BCl<sub>3</sub>.SMe<sub>2</sub> (2M in DCM, 7.00 equiv.) and the reaction was stirred at room temperature for 8 h. The mixture was concentrated *in vacuo*. The residue was re-suspended in TFA:H<sub>2</sub>O (3:1, 4 mL) and the reaction was stirred overnight at room temperature. The mixture was concentrated *in vacuo*. The crude product was purified by preparative HPLC to afford the desired product as a colourless solid.

**Preparation of (2S)-2-amino-1-[[[(2R, 3S, 4R, 5R)-5-(6-amino-2-chloro-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy)sulfonyl]amino]-3-hydroxypropan-1-one (2).** Preparation was via general method D using tert-butyl N-[(2S)-1-[[[(3aR, 4R, 6R, 6aR)-6-(6-amino-2-chloro-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methoxy)sulfonyl]amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (**24**) (0.30 g, 0.43 mmol) to afford the desired product as a colourless powder (50.4 mg, 0.11 mmol, 25%) m.p.: 121.3 °C (Decomp); *R*<sub>f</sub>: Baseline (9:1 DCM-MeOH); δ<sub>H</sub> (500 MHz, DMSO-d<sub>6</sub>): 8.48 (1H, s, 6-HAr), 8.42 (2H, brs, NH<sub>2</sub>), 7.27 (2H, brs, NH<sub>2</sub>), 7.00 (1H, brs, NH), 6.20 (1H, d, J 8.2, 2-HFuryl), 4.99 (1H, brs, OH) 4.75 (1H, apps, 3-HFuryl), 4.62-4.48 (3H, m, 4-HFuryl, 5-HFuryl and OH), 4.27 (1H, dd, J 10.1 and 5.9, CH<sub>2</sub>\*O), 4.19 (1H, brs, OH), 4.02 (1H, dd, J 10.1 and 6.2, CH<sub>2</sub>\*O), 3.52 (3H, m, CH<sub>2</sub>\*chiral and CHChiral); δ<sub>C</sub> (125 MHz, DMSO-d<sub>6</sub>): 179.3 (C=O), 157.1 (C4Ar), 153.5 (C2Ar and C8Ar), 140.4 (C6Ar), 120.0 (C9Ar), 89.4 (C2Furyl), 84.4 (C5Furyl), 75.3 (C3Furyl), 74.8 (C4Furyl), 66.0 (CH<sub>2</sub>O), 64.7 (CH<sub>2</sub>Chiral), 52.9 (CHChiral); ν<sub>max</sub>/cm<sup>-1</sup> (solid): 3321, 3125, 2784, 1673, 1592, 1358; HPLC: T<sub>r</sub> = 2.26 (100% rel. area); *m/z* (ES): No mass ion found [α]<sub>D</sub> = 28.8° (c 0.1, MeOH).

**Preparation of (2S)-2-amino-1-[[[(2R, 3S, 4R, 5R)-5-(6-amino-2-iodo-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy)sulfonyl]amino]-3-hydroxypropan-1-one (3).** Preparation was via general method D using tert-butyl N-[(2S)-1-[[[(3aR, 4R, 6R, 6aR)-6-(6-amino-2-iodo-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methoxy)sulfonyl]amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (**25**) (50 mg, 0.06 mmol) to afford the desired product as a colourless powder

(4.0 mg, 0.07 mmol, 12%) m.p.: 135.2 °C (Decomp);  $R_f$ : Baseline (9:1 DCM-MeOH);  $\delta_H$  (400 MHz, DMSO-d<sub>6</sub>): 8.48 (1H, s, 6-HAr), 8.43 (2H, brs, NH<sub>2</sub>), 7.26 (1H, brs NH<sub>2</sub>) 7.00 (1H, brs, NH), 6.19 (1H, d, J 5.4, 2-HFuryl), 4.99 (1H, brs, OH), 4.74 (1H, apps, 3-HFuryl), 4.59-4.50 (3H, m, 4-HFuryl, 5-HFuryl and OH), 4.26 (1H, dd, J 10.2 and 5.9, CH<sub>2</sub>\*O), 4.18 (1H, brs, OH), 4.04 (1H, dd, J 10.0 and 5.8, CH<sub>2</sub>\*O), 3.60-3.49 (3H, m, CH<sub>2</sub>\*chiral and CH Chiral);  $\delta_C$  (100 MHz, DMSO-d<sub>6</sub>): 179.1 (C=O), 157.5 (C4Ar), 151.0 (C8Ar), 140.3 (C6Ar), 126.9 (C2Ar), 118.1 (C9Ar), 89.4 (C2Furyl), 83.9 (C5Furyl), 75.4 (C3Furyl), 74.8 (C4Furyl), 65.7 (CH<sub>2</sub>O), 64.4 (CH<sub>2</sub>Chiral), 51.9 (CHChiral);  $\nu_{max}$ /cm<sup>-1</sup> (solid): 3321, 3125, 2784, 1673, 1592, 1358; HPLC:  $T_r$  = 2.30 (95% rel. area);  $m/z$  (ES): (Found: [M-H]<sup>-</sup>, 558.0. C<sub>13</sub>H<sub>18</sub>IN<sub>7</sub>O<sub>8</sub>S requires [M-H], 558.0.  $[\alpha]_D$  = 28.4° (c 0.1, MeOH).

**Preparation of (2S)-2-amino-1-([[(2R, 3S, 4R, 5R)-5-(6-amino-2-phenyl-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy)sulfonyl]amino]-3-hydroxypropan-1-one (4).** Preparation was via general method D using tert-butyl N-[(2S)-1-([[(3aR, 4R, 6R, 6aR)-6-(6-amino-2-phenyl-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methoxy)sulfonyl]amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (**26**) (0.20 g, 0.27 mmol) to afford the desired product as a colourless powder (54.0 mg, 0.11 mmol, 39%) m.p.: 143.2 (Decomp);  $R_f$ : Baseline (9:1 DCM-MeOH);  $\delta_H$  (400 MHz, DMSO-d<sub>6</sub>): 8.47 (1H, s, 6-HAr), 8.43 (2H, dd, J 8.0 and 1.6, 6'-HAr and 2'-HAr), 8.20 (1H, s, NH), 7.91 (2H, brs, NH<sub>2</sub>), 7.58-7.48 (3H, m, 3'-HAr, 4'-HAr and 5'-HAr), 6.09 (1H, d, J 5.9, 2-HFuryl), 4.77-4.70 (1H, m, 3-HFuryl), 4.34-4.28 (2H, m, 4-HFuryl and CH<sub>2</sub>\*O), 4.22-4.16 2H, m, 5-HFuryl and CH<sub>2</sub>\*O), 3.87 (1H, dd, J 11.2 and 3.7, CH<sub>2</sub>\*Chiral) 3.69 (1H, dd, J 11.2 and 7.3, CH<sub>2</sub>\*Chiral), 3.60-3.56 (1H, m, ChiralH);  $\delta_C$  (100 MHz, DMSO-d<sub>6</sub>): 172.0 (C=O), 163.5 (C2Ar), 155.9 (C4Ar), 150.8 (C8Ar), 140.5 (C6Ar), 137.8 (C1'Ar), 130.1 (C4'Ar), 128.7 (C5'Ar and C3'Ar), 128.2 (C6'Ar and C2'Ar), 119.1 (C9Ar), 87.1 (C2Furyl), 82.8 (C5Furyl), 73.8 (C3Furyl), 71.3 (C4Furyl), 68.5 (CH<sub>2</sub>O), 61.0 (CH<sub>2</sub>Chiral), 57.6 (Chiral H);  $\nu_{max}$ /cm<sup>-1</sup> (solid): 3367, 3070, 1673, 1598; HPLC:  $T_r$  = 1.89 (100% rel. area);  $m/z$  (ES): (Found: [M+H]<sup>+</sup>, 510.1405. C<sub>19</sub>H<sub>23</sub>N<sub>7</sub>O<sub>8</sub>S requires [M+H], 510.1402).  $[\alpha]_D$  = 25.7° (c 0.1, MeOH).

**Preparation of (2S)-2-amino-1-([[(2R, 3S, 4R, 5R)-5-(6-amino-2-propenyl-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy)sulfonyl]amino]-3-hydroxypropan-1-one (5).** Preparation was via general method D using tert-butyl N-[(2S)-1-([[(3aR, 4R, 6R, 6aR)-6-(6-amino-2-propenyl-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methoxy)sulfonyl]amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (**27**) (65 mg, 0.09 mmol) to afford the desired product as a colourless powder (22.5 mg, 0.05 mmol, 53%) m.p.: 115.3 (Decomp);  $R_f$ : Baseline (9:1 DCM-MeOH);  $\delta_H$  (500 MHz, DMSO-d<sub>6</sub>): 8.50 (1H, s, 6-HAr), 8.15 (1H, s, NH), 7.88 (2H, brs, NH<sub>2</sub>), 7.09 (1H, dd, J 15.4 and 6.7, 2-HPropyl), 6.41 (1H, d, J 15.4, 1-HPropyl), 5.95 (1H, d, J 5.9, 2-HFuryl), 4.63 (1H, app t, J 5.9, 3-HFuryl), 4.28-4.09 (4H, m, 4-HFuryl, 5-HFuryl and CH<sub>2</sub>O), 3.83 (1H, dd, J 11.1 and 3.6, CH<sub>2</sub>\* Chiral), 3.65 (1H, dd, J 11.1

and 7.0, CH<sub>2</sub>\* Chiral), 3.56-3.50 (1H, m, Chiral H), 2.88 (2H, app d, J 5.3, NH<sub>2</sub>), 1.98 (3H, d, J 6.7, CH<sub>3</sub>);  $\delta_C$  (125 MHz, DMSO-d<sub>6</sub>): 172.2 (C=O), 163.5 (C2Ar), 153.8 (C4Ar), 150.9 (C8Ar), 145.9 (C6Ar), 122.3 (C2Pro), 119.4 (C9Ar), 117.9 (C1Pro), 93.7 (C2furyl), 83.4 (C5Furyl), 75.4 (C3Furyl), 70.5 (C4Furyl), 70.2 (CH<sub>2</sub>O), 57.3 (CH<sub>2</sub> Chiral), 54.8 (Chiral H), 19.1 (CH<sub>3</sub>);  $\nu_{\max}$ /cm<sup>-1</sup> (solid): 3106, 2942, 1668, 1595, 1182; HPLC: T<sub>r</sub> = 0.81 (100% rel. area); *m/z* (ES): (Found: [M+H]<sup>+</sup>, 474.1406. C<sub>16</sub>H<sub>23</sub>N<sub>7</sub>O<sub>8</sub>S requires [M+H], 474.1402). [ $\alpha$ ]<sub>D</sub> = 27.0° (c 0.1, MeOH).

**Preparation of (2S)-2-amino-1-([[(2R, 3S, 4R, 5R)-5-(6-amino-2-(furan-2-yl)-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy)sulfonyl]amino]-3-hydroxypropan-1-one (6).** Preparation was via general method D using tert-butyl N-[(2S)-1-([[(3aR, 4R, 6R, 6aR)-6-(6-amino-2-(furan-2-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methoxy)sulfonyl]amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (**28**) (100 mg, 0.14 mmol) to afford the desired product as a colourless powder (14.6 mg, 0.03 mmol, 21%) m.p.: 117.2 (Decomp); *R*<sub>f</sub>: Baseline (9:1 DCM-MeOH);  $\delta_H$  (500 MHz, DMSO-d<sub>6</sub>): 8.54 (1H, brs, NH), 8.34 (2H, brs, NH<sub>2</sub>), 8.14 (1H, s, 6-HAr), 7.96 (1H, app s, 3'-HAr), 7.46 (1H, app t, J 10.1, 5'-HAr), 6.76 (1H, dd, J 10.1 and 8.5, 4'-HAr), 6.02 (1H, d, J 5.2, 2-HFuryl), 4.65 (1H, d, J 5.2, 3-HFuryl), 4.52-4.45 (2H, m, CH<sub>2</sub>O), 4.29 (1H, app s, 4-HFuryl), 4.20 (1H, app s, 5-HFuryl), 3.89-3.75 (3H, m, CH<sub>2</sub> Chiral and Chiral H);  $\delta_C$  (125 MHz, DMSO-d<sub>6</sub>): 172.0 (C=O), 158.1 (C2Ar), 156.1 (C4Ar), 152.5 (C1'Ar), 150.6 (C8Ar), 145.3 (C3'Ar), 141.2 (C6Ar), 117.9 (C9Ar), 113.9 (C5'Ar), 113.2 (C4'Ar), 88.2 (C2Furyl), 84.6 (C5Furyl), 73.4 (C3Furyl), 71.8 (C4Furyl), 60.6 (CH<sub>2</sub>O), 54.9 (Chiral C), 45.2 (CH<sub>2</sub> Chiral);  $\nu_{\max}$ /cm<sup>-1</sup> (solid): 3089, 1689, 1595, 1477, 1383; HPLC: T<sub>r</sub> = 2.04 (97% rel. area); *m/z* (ES): (Found: [M+H]<sup>+</sup>, C<sub>17</sub>H<sub>21</sub>N<sub>7</sub>O<sub>9</sub>S requires [M+H], 500.1194.) [ $\alpha$ ]<sub>D</sub> = 22.9° (c 0.1, MeOH).

**Preparation of (2S)-2-amino-1-([[(2R, 3S, 4R, 5R)-5-(6-amino-2-(thiophen-3-yl)-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy)sulfonyl]amino]-3-hydroxypropan-1-one (7).** Preparation was via general method D using tert-butyl N-[(2S)-1-([[(3aR, 4R, 6R, 6aR)-6-(6-amino-2-(thiophen-3-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methoxy)sulfonyl]amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (**29**) (0.30 g, 0.40 mmol) to afford the desired product as a colourless powder (27.5 mg, 0.05 mmol, 13%) m.p.: 108.3 °C (Decomp); *R*<sub>f</sub>: Baseline (9:1 DCM-MeOH);  $\delta_H$  (500 MHz, DMSO-d<sub>6</sub>): 8.46 (1H, brs, NH), 8.34 (1H, s, 2'-HAr), 8.14 (1H, s, 6-HAr), 8.00 (2H, brs, NH<sub>2</sub>), 7.87-7.78 (1H, m, 4'-HAr), 7.68-7.59 (1H, m, 5'-HAr), 6.00 (1H, d, J 5.5, 2-HFuryl), 4.70 (1H, t, J 5.5, 3-HFuryl), 4.37-4.22 (4H, m, CH<sub>2</sub>O, 4-HFuryl and 5-HFuryl), 3.83-3.65 (3H, m, CH<sub>2</sub> chiral and chiral H);  $\delta_C$  (125 MHz, DMSO-d<sub>6</sub>): 172.0 (C=O), 163.5 (C2Ar), 156.0 (C4Ar), 150.7 (C8Ar), 140.1 (C6Ar), 127.8 (C4'Ar), 127.1 (C5'Ar), 120.0 (C2'Ar), 119.2 (C9Ar), 116.8 (C1'Ar), 88.2 (C2Furyl), 84.2 (C5furyl), 73.7 (C3Furyl), 71.8 (C4Furyl), 60.8 (CH<sub>2</sub>O), 57.2 (Chiral C), 45.4 (CH<sub>2</sub> chiral);  $\nu_{\max}$ /cm<sup>-1</sup> (solid): 3096,

1685, 1588, 1402, 1275; HPLC:  $T_r$  = 2.04 (100% rel. area);  $m/z$  (ES): (Found:  $[M+H]^+$ , 516.0972.  $C_{17}H_{21}N_7O_8S_2$  requires  $[M+H]$ , 516.0966.)  $[\alpha]_D = 20.7^\circ$  (c 0.1, MeOH).

**Preparation of (2S)-2-amino-1-[[[(2R, 3S, 4R, 5R)-5-(6-amino-2-(pyridine-3-yl)-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy)sulfonyl]amino]-3-hydroxypropan-1-one (8).** Preparation was via general method D using tert-butyl N-[(2S)-1-[[[(3aR, 4R, 6R, 6aR)-6-(6-amino-2-(pyridine-3-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methoxy)sulfonyl]amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (**30**) (100 mg, 0.13 mmol) to afford the desired product as a colourless powder (23.8 mg, 0.05 mmol, 36%) m.p.: 120.2 °C (Decomp);  $R_f$ : Baseline (9:1 DCM-MeOH);  $\delta_H$  (500 MHz, DMSO- $d_6$ ): 9.50 (1H, s, 2'-HAr), 8.95-8.75 (2H, m, 6'-HAr and 4'-HAr), 8.47 (1H, s, 6-HAr), 7.93 (2H, brs,  $NH_2$ ), 7.54-7.50 (1H, m, 5'-HAr), 6.04 (1H, d, J 6.0 2-HFuryl), 4.82-4.72 (1H, m, 3-HFuryl), 4.37-4.23 (2H, m,  $CH_2^*O$  and 4-HFuryl), 4.18 (2H, app d, J 8.9, 5-HFuryl and  $CH_2^*O$ ), 3.82 (1H, d, J 8.0  $CH_2$  chiral), 3.73-3.60 (1H, m,  $CH_2$  chiral), 3.58-3.54 (1H, m, Chiral H);  $\delta_C$  (125 MHz, DMSO- $d_6$ ): 171.9 (C=O), 160.3 (C2Ar), 156.1 (C4Ar), 151.0 (C4'Ar), 150.8 (C8Ar), 150.7 (C2'Ar), 141.2 (C6Ar), 138.6 (C2'Ar), 135.6 (C6'Ar), 133.4 (C1'Ar), 125.2 (C5'Ar), 119.4 (C9Ar), 87.9 (C2Furyl), 82.9 (C5Furyl), 73.7 (C3Furyl), 71.2 (C4Furyl), 68.7 ( $CH_2O$ ), 60.8 ( $CH_2$  chiral), 57.5 (Chiral C);  $\nu_{max}/cm^{-1}$  (solid): 3317, 3118, 1633, 1587, 1587, 1382; HPLC:  $T_r$  = 1.99 (100% rel. area);  $m/z$  (ES): (Found:  $[M+H]^+$ , 511.1356.  $C_{18}H_{22}N_8O_8S$  requires  $[M+H]$ , 511.1356.  $[\alpha]_D = 19.3^\circ$  (c 0.1, MeOH).

**Preparation of 2'3'-O-Isopropylidene-2-chloroadenosine (9).** 2-Chloroadenosine (0.20 g, 0.66 mmol, 1.0 equiv.) and p-toluenesulfonic acid mono hydrate (1.26 g, 6.6 mmol, 10 equiv.) were dissolved in acetone (100 mL) under an atmosphere of nitrogen. The reaction was stirred at room temperature overnight. While cooling in an ice bath a saturated  $NaHCO_3$  solution (100 mL) was added to the reaction mixture until the pH of the solution was slightly basic. The acetone was removed *in vacuo*. The remaining aqueous solution was extracted with EtOAc (3  $\times$  50 mL). The combined organics were dried ( $MgSO_4$ ) and concentrated *in vacuo*. The desired product was isolated as a colourless solid (203 mg, 0.59 mmol, 90%); m.p.: 184.2-185.6 °C;  $R_f$ : 0.67 (9:1 Chloroform-MeOH);  $\delta_H$  (500 MHz, DMSO- $d_6$ ): 8.37 (1H, s, 6-HAr), 7.87 (2H, brs,  $NH_2$ ), 6.07 (1H, d, J 2.8 2-HFuryl), 5.29 (1H, dd, J 6.2 and 2.8, 3-HFuryl), 5.09 (1H, app t, J 5.4, OH), 4.95 (1H, dd, J 6.2 and 2.8, 4-HFuryl), 4.22 (1H, dd, J 6.2 and 5.4, 5-HFuryl), 3.65-3.50 (2H, m,  $CH_2$ ), 1.56 (3H, s,  $CH_3a$ ), 1.34 (3H, s,  $CH_3b$ );  $\delta_C$  (125 MHz, DMSO- $d_6$ ): 158.1 (C4Ar), 153.0 (C2Ar), 149.9 (C8Ar), 139.9 (C6Ar), 128.2 (C5Ar), 119.0 (C9Ar), 113.1 (Acetyl C), 89.4 (C2Furyl), 86.7 (C5Furyl), 83.4 (C3Furyl), 81.2 (C4Furyl), 61.5 ( $CH_2$ ), 37.0 ( $CH_3a$ ), 25.2 ( $CH_3b$ );  $\nu_{max}/cm^{-1}$  (solid): 3473, 3299, 1761, 1651, 1381; HPLC:  $T_r$  = 2.26 (100% rel. area);  $m/z$  (ES): (Found:  $[M+H]^+$ , 342.0965.  $C_{13}H_{16}ClN_5O_4$  requires  $[M+H]$ , 342.0964.  $[\alpha]_D = -115.5^\circ$  (c 0.1, MeOH).

**Preparation of 2'3'-O-Isopropylidene-2-iodoadenosine (10).** 2-iodoadenosine (1.00 g, 2.54 mmol, 1.0 equiv.) and p-toluenesulfonic acid mono hydrate (4.83 g, 25.4 mmol, 10 equiv.) were dissolved in acetone (200 mL) under an atmosphere of nitrogen. The reaction was stirred at room temperature overnight. While cooling in an ice bath a saturated NaHCO<sub>3</sub> solution (200 mL) was added to the reaction mixture until the pH of the solution was slightly basic. The acetone was removed *in vacuo*. The remaining aqueous solution was extracted with EtOAc (3 × 100 mL). The combined organics were dried (MgSO<sub>4</sub>) and concentrated *in vacuo*. The desired product was isolated as a colourless solid (0.94 g, 2.17 mmol, 86%); m.p.: 182.4-184.1 °C; *R*<sub>f</sub>: 0.69 (9:1 Chloroform-MeOH); δ<sub>H</sub> (400 MHz, DMSO-d<sub>6</sub>): 8.34 (1H, s, 6-HAr), 7.83 (2H, brs, NH<sub>2</sub>), 6.06 (1H, d, J 4.0, 2-HFuryl), 5.30 (1H, dd, J 6.2 and 4.0, 3-HFuryl), 5.09 (1H, app t, J 5.2, OH), 4.96 (1H, dd, J 6.2 and 3.9, 4-HFuryl), 4.20 (1H, d, J 3.9, 5-HFuryl), 3.52 (2H, app s, CH<sub>2</sub>), 1.56 (3H, s, CH<sub>3a</sub>), 1.37 (3H, s, CH<sub>3b</sub>); δ<sub>C</sub> (100 MHz, DMSO-d<sub>6</sub>): 158.2 (C4Ar), 156.4 (C8Ar), 149.8 (C6Ar), 121.4 (C9Ar), 119.3 (C2Ar), 113.6 (Acetyl C), 89.6 (C2Furyl), 87.3 (C5Furyl), 84.0 (C3Furyl), 81.7 (C4Furyl), 62.0 (CH<sub>2</sub>), 27.5 (CH<sub>3a</sub>), 25.7 (CH<sub>3b</sub>); ν<sub>max</sub>/cm<sup>-1</sup> (solid): 3473, 3299, 1761, 1651, 1381; HPLC: T<sub>r</sub> = 2.30 (100% rel. area); *m/z* (ES): (Found: [M+Na]<sup>+</sup>, 455.8. C<sub>13</sub>H<sub>16</sub>IN<sub>5</sub>O<sub>4</sub> requires [M+Na], 455.8. [α]<sub>D</sub> = -102.0° (c 0.1, MeOH).

**Preparation of 2'3'-O-Isopropylidene-2-phenyladenosine (11).** Preparation was via general method A using 2'3'-O-Isopropylidene-2-chloroadenosine (9) (0.50 g, 1.46 mmol) and phenyl boronic acid (0.71 g, 5.85 mmol) to afford the desired product as a colourless powder (0.43 g, 1.12 mmol, 76%); m.p.: 166.2-168.1 °C; *R*<sub>f</sub>: 0.58 (9:1 Chloroform-MeOH); δ<sub>H</sub> (500 MHz, DMSO-d<sub>6</sub>): 8.36 (3H, app s, 6-HAr, 2'-HAr and 6'-HAr), 7.46 (3H, app s, 4'-HAr, 3'-HAr and 5'-HAr), 7.39 (2H, brs, NH<sub>2</sub>), 6.26 (1H, app s, 2-HFuryl), 5.52 (1H, app s, 3-HFuryl), 5.10 (1H, app s, 4-HFuryl), 5.04 (1H, app s, OH), 4.22 (1H, app s, 4-HFuryl), 3.69-3.51 (2H, m, CH<sub>2</sub>), 1.59 (3H, s, CH<sub>3a</sub>), 1.37 (3H, s, CH<sub>3b</sub>); δ<sub>C</sub> (125 MHz, DMSO-d<sub>6</sub>): 158.5 (C2Ar), 156.4 (C4Ar), 150.4 (C8Ar), 140.8 (C6Ar), 138.8 (C1'Ar), 134.4 (C4'Ar), 128.7 (C3'Ar and C5'Ar), 128.2 (C2'Ar and C6'Ar), 118.7 (C9Ar), 113.6 (Acetyl C), 89.5 (C2Furyl), 87.2 (C5Furyl), 83.8 (C3Furyl), 81.9 (C4Furyl), 62.0 (CH<sub>2</sub>), 27.6 (CH<sub>3a</sub>), 25.7 (CH<sub>3b</sub>); ν<sub>max</sub>/cm<sup>-1</sup> (solid): 3314, 3157, 1655, 1596, 1372; HPLC: T<sub>r</sub> = 2.24 (100% rel. area); *m/z* (ES): (Found: [M+H]<sup>+</sup>, 384.1676. C<sub>19</sub>H<sub>21</sub>N<sub>5</sub>O<sub>4</sub> requires [M+H], 384.1666. [α]<sub>D</sub> = -1.4° (c 0.1, MeOH).

**Preparation of 2'3'-O-Isopropylidene-2-propenyladenosine (12).** Preparation was via general method A using 2'3'-O-Isopropylidene-2-chloroadenosine (9) (0.50 g, 1.46 mmol) and transpropenyl boronic acid (0.50 g, 5.85 mmol) to afford the desired product as a colourless powder (0.17 g, 0.50 mmol, 34%); m.p.: 81.6-83.4 °C; *R*<sub>f</sub>: 0.58 (9:1 Chloroform-MeOH); δ<sub>H</sub> (500 MHz, DMSO-d<sub>6</sub>): 8.29 (1H, s, 6-HAr), 7.22 (2H, brs, NH<sub>2</sub>), 6.92 (1H, dd, J 15.2 and 7.2, 2-Hpropyl), 6.33 (1H, d, J 15.2, 1-Hpropyl), 6.13 (1H, app s, 2-HFuryl), 5.34 (1H, app s, 3-HFuryl), 5.26 (1H, t, J 5.4, OH), 5.02 (1H, app s, 4-HFuryl), 4.22 (1H, app s, 5-HFuryl), 3.62-3.49 (2H, m, CH<sub>2</sub>), 1.90 (3H, d, J 7.2, CH<sub>3</sub>propyl), 1.56 (3H, s, CH<sub>3a</sub>), 1.35 (3H, s, CH<sub>3b</sub>);

$\delta_C$  (125 MHz, DMSO- $d_6$ ): 158.8 (C2Ar), 156.2 (C4Ar), 149.9 (C8Ar), 140.3 (C6Ar), 134.0 (C2Pro), 131.9 (C1Pro), 119.1 (C9Ar), 89.9 (C2Furyl), 86.9 (C5Furyl), 83.6 (C3Furyl), 81.9 (C4Furyl), 62.2 (CH<sub>2</sub>), 27.6 (CH<sub>3a</sub>), 25.7 (CH<sub>3b</sub>), 18.3 (CH<sub>3</sub>Pro);  $\nu_{max}/\text{cm}^{-1}$  (solid): 3451, 3310, 3162, 1657, 1575, 1373; HPLC:  $T_r$  = 2.11 (72% rel. area);  $m/z$  (ES): (Found:  $[M+H]^+$ , 348.3.  $C_{16}H_{21}N_5O_4$  requires  $[M+H]$ , 348.5.  $[\alpha]_D = -50.2^\circ$  (c 0.1, MeOH).

**Preparation of 2'3'-O-Isopropylidene-2-(furan-2-yl)adenosine (13).** Preparation was via general method A using 2'3'-O-Isopropylidene-2-chloroadenosine (**9**) (0.50 g, 1.46 mmol) and 2-furyl boronic acid (0.66 g, 5.85 mmol) to afford the desired product as a colourless powder (0.25 g, 0.67 mmol, 46%); m.p.: 96.3-98.2 °C;  $R_f$ : 0.59 (9:1 Chloroform-MeOH);  $\delta_H$  (500 MHz, DMSO- $d_6$ ): 8.35 (1H, s, 6-HAr), 7.83 (1H, 5'-HAr), 7.44 (2H, brs, NH<sub>2</sub>), 7.13 (1H, s, 3'-HAr), 6.65 (1H, s, 4'-HAr), 6.20 (1H, app s, 2-HFuryl), 5.40 (1H, app s, 3-HFuryl), 5.10 (1H, app s, 4-HFuryl), 5.05 (1H, app s, OH), 4.22 (1H, app s, 5-HFuryl), 3.67-3.52 (2H, m, CH<sub>2</sub>), 1.55 (3H, s, CH<sub>3a</sub>), 1.35 (3H, s, CH<sub>3b</sub>);  $\delta_C$  (125 MHz, DMSO- $d_6$ ): 156.8 (C2Ar), 156.0 (C4Ar), 152.6 (C2'Ar), 149.3 (C8Ar), 144.2 (C5'Ar), 140.2 (C6Ar), 117.9 (C9Ar), 113.0 (Acetyl C), 112.0 (C3'Ar), 111.4 (C4'Ar), 89.0 (C2Furyl), 86.8 (C5Furyl), 83.3 (C3Furyl), 81.5 (C4Furyl), 61.6 (CH<sub>2</sub>), 27.9 (CH<sub>3a</sub>), 25.2 (CH<sub>3b</sub>);  $\nu_{max}/\text{cm}^{-1}$  (solid): 3419, 3311, 2987, 2938, 1639, 1547, 1369; HPLC:  $T_r$  = 2.20 (57% rel. area);  $m/z$  (ES): (Found:  $[M+H]^+$ , 374.1462.  $C_{17}H_{19}N_5O_5$  requires  $[M+H]$ , 374.1459.  $[\alpha]_D = -44.8^\circ$  (c 0.1, MeOH).

**Preparation of 2'3'-O-Isopropylidene-2-(thiophen-3-yl)adenosine (14).** Preparation was via general method A using 2'3'-O-Isopropylidene-2-chloroadenosine (**9**) (0.50 g, 1.46 mmol) and 3-thienyl boronic acid (0.75 g, 5.85 mmol) to afford the desired product as a colourless powder (0.46 g, 1.17 mmol, 80%); m.p.: 100.1-102.0 °C;  $R_f$ : 0.62 (9:1 Chloroform-MeOH);  $\delta_H$  (500 MHz, DMSO- $d_6$ ): 8.34 (1H, s, 6-HAr), 8.18 (1H, dd, J 3.1 and 1.2, 2'-HAr), 7.79 (1H, dd, J 5.0 and 1.2, 4'-HAr), 7.42 (1H, dd, J 5.0 and 3.1, 5'-H), 7.36 (2H, brs, NH<sub>2</sub>), 6.23 (1H, d, J 6.2, 2-HFuryl), 5.49 (1H, dd, J 6.2 and 2.7, 3-HFuryl), 5.11 (1H, dd, J 6.2 and 2.7, 4-HFuryl), 4.22 (1H, t, J 5.5 and 2.7, 5-HFuryl), 3.59 (2H, dd, J 11.5 and 5.5, CH<sub>2</sub>), 1.57 (3H, s, CH<sub>3a</sub>), 1.36 (3H, s, CH<sub>3b</sub>);  $\delta_C$  (125 MHz, DMSO- $d_6$ ): 173.2 (C2Ar), 155.8 (C4Ar), 140.3 (C6Ar), 127.4 (C4'Ar), 125.1 (C5'Ar), 120.0 (C2'Ar), 119.2 (C9Ar), 116.7 (C1'Ar), 114.2 (AcetylC), 90.6 (C2Furyl), 87.3 (C5Furyl), 83.6 (C3Furyl), 81.2 (C4Furyl), 61.8 (CH<sub>2</sub>), 26.7 (CH<sub>3a</sub>), 25.0 (CH<sub>3b</sub>);  $\nu_{max}/\text{cm}^{-1}$  (solid): 3253, 2939, 1633, 1586, 1344; HPLC:  $T_r$  = 2.19 (79% rel. area);  $m/z$  (ES): (Found:  $[M+H]^+$ , 365.1058.  $C_{17}H_{19}N_5O_4S$  requires  $[M+H]$ , 365.1051.  $[\alpha]_D = -6.6^\circ$  (c 0.1, MeOH).

**Preparation of 2'3'-O-Isopropylidene-2-(pyridine-3-yl)adenosine (15).** Preparation was via general method A using 2'3'-O-Isopropylidene-2-chloroadenosine (**9**) (0.50 g, 1.46 mmol) and 3-pyridine boronic acid (0.71 g, 5.85 mmol) to afford the desired product as a colourless powder (0.29 g, 0.75 mmol, 51%);



m.p.: 122.4-124.2 °C;  $R_f$ : 0.53 (9:1 Chloroform-MeOH);  $\delta_H$  (500 MHz, DMSO-d<sub>6</sub>): 9.49 (1h, s, 2'-HAr), 8.67-8.61 (2H, m, 6'-HAr and 4'-HAr), 8.40 (1H, s, 6-HAr), 7.52 (3H, brs, NH<sub>2</sub> and 5'-HAr), 6.26 (1H, d, J 2.7, 2-HFuryl), 5.52 (1H, dd, J 6.1 and 2.7, 3-HFuryl), 5.10 (1H, dd, J 6.1 and 2.9, 4-HFuryl), 5.05 (1H, t, J 5.5, OH), 4.23 (1H, dd, J 8.2 and 5.3, 5-HFuryl), 3.62-3.51 (2H, m, CH<sub>2</sub>), 1.57 (3H, s, CH<sub>3</sub>a), 1.36 (3H, s, CH<sub>3</sub>b);  $\delta_C$  (125 MHz, DMSO-d<sub>6</sub>): 160.3 (C2Ar), 156.5 (C4Ar), 150.8 (C4Pip), 150.2 (C8Ar), 149.5 (C2Pip), 141.1 (C6Ar), 134.5 (C6Pip), 134.1 (C1Pip), 124.0 (C5Pip), 119.0 (C9Ar), 113.5 (Acetyl C), 89.7 (C2Furyl), 87.2 (C5Furyl), 83.8 (C3furyl), 81.9 (C4Furyl), 62.0 (CH<sub>2</sub>), 27.6 (CH<sub>3</sub>a), 25.7 (CH<sub>3</sub>b);  $\nu_{max}$ /cm<sup>-1</sup> (solid): 3322, 1632, 1572, 1375; HPLC:  $T_r$  = 1.99 (100% rel. area);  $m/z$  (ES): (Found: [M+H]<sup>+</sup>, 385.1626. C<sub>18</sub>H<sub>20</sub>N<sub>6</sub>O<sub>4</sub> requires [M+H], 385.1619.  $[\alpha]_D$  = -1.4° (c 0.1, MeOH).

**Preparation of [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-chloro-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl sulfamate (16).** Preparation was *via* general method B using 2'3'-O-Isopropylidene-2-chloroadenosine (**9**) (0.17 g, 0.49 mmol, 1.0 equiv.) to afford the desired product as a colourless glassy solid (0.19 g, 0.46 mmol, 94%) m.p.: 69.4-71.7 °C;  $R_f$ : 0.46 (9:1 Chloroform-MeOH);  $\delta_H$  (500 MHz, DMSO-d<sub>6</sub>): 8.33 (1H, s, 6-HAr), 7.90 (2H, brs, NH<sub>2</sub>), 7.60 (2H, brs, SNH<sub>2</sub>), 6.18 (1H, d, J 2.5, 2-HFuryl), 5.36 (1H, dd, J 6.2 and 2.3, 3-HFuryl), 5.03 (1H, dd, J 6.2 and 3.4, 4-HFuryl), 4.43 (1H, dd, J 9.0 and 3.4, 5-HFuryl), 4.23 (2H, ddd, J 17.2, 9.0 and 3.4, CH<sub>2</sub>), 1.57 (3H, s, CH<sub>3</sub>a), 1.36 (3H, s, CH<sub>3</sub>b);  $\delta_C$  (125 MHz, DMSO-d<sub>6</sub>): 156.9 (C4Ar), 153.2 (C2Ar), 149.8 (C8Ar), 139.9 (C6Ar), 118.1 (C9Ar), 113.7 (C Acetyl), 88.8 (C2Furyl), 83.7 (C4Furyl), 83.4 (C3Furyl), 80.9 (C4Furyl), 68.1 (CH<sub>2</sub>), 26.9 (CH<sub>3</sub>a), 25.2 (CH<sub>3</sub>b);  $\nu_{max}$ /cm<sup>-1</sup> (solid): 3321, 3171, 1594, 1206; HPLC:  $T_r$  = 2.30 (95% rel. area);  $m/z$  (ES): (Found: [M+H]<sup>+</sup>, 421.0695. C<sub>13</sub>H<sub>17</sub>ClN<sub>6</sub>O<sub>6</sub>S requires [M+H], 421.0692.  $[\alpha]_D$  = -20.2° (c 0.1, MeOH).

**Preparation of [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-iodo-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl sulfamate (17).** Preparation was *via* general method B using 2'3'-O-Isopropylidene-2-iodoadenosine (**10**) (0.20 g, 0.46 mmol) to afford the desired product as a colourless glassy solid (0.20 g, 0.39 mmol, 85%) m.p.: 70.2-71.7 °C;  $R_f$ : 0.48 (9:1 Chloroform-MeOH);  $\delta_H$  (500 MHz, DMSO-d<sub>6</sub>): 8.24 (1H, s, 6-HAr), 7.80 (2H, brs, NH<sub>2</sub>), 7.59 (2H, brs, SNH<sub>2</sub>), 6.18 (1H, d, J 4.0, 2-HFuryl), 5.33 (1H, dd, J 6.1 and 4.0, 3-HFuryl), 5.02 (1H, dd, J 6.1 and 3.8), 4.43 (1H, app d, J 3.8, 5-HFuryl), 4.28-4.12 (2H, m, CH<sub>2</sub>), 1.63 (3H, s, CH<sub>3</sub>a), 1.38 (3H, s, CH<sub>3</sub>b);  $\delta_C$  (100 MHz, DMSO-d<sub>6</sub>): 156.5 (C4Ar), 156.4 (C8Ar), 149.7 (C6Ar), 121.5 (C9Ar), 119.4 (C2Ar), 114.2 (Acetyl C), 89.1 (C2Furyl), 84.3 (C5Furyl), 84.1 (C3Furyl), 81.4 (C4Furyl), 68.5 (CH<sub>2</sub>), 27.4 (CH<sub>3</sub>a), 25.7 (CH<sub>3</sub>b);  $\nu_{max}$ /cm<sup>-1</sup> (solid): 3321, 3171, 1594, 1206; HPLC:  $T_r$  = 2.35 (100% rel. area);  $m/z$  (ES): (Found: [M+Na]<sup>+</sup>, 442.9. C<sub>13</sub>H<sub>17</sub>IN<sub>6</sub>O<sub>6</sub>S requires [M+Na], 442.9.  $[\alpha]_D$  = -21.9° (c 0.1, MeOH).

**Preparation of [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-phenyl-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl sulfamate (18).** Preparation was *via* general method B using 2'3'-O-Isopropylidene-2-phenyladenosine (**11**) (0.40 g, 1.04 mmol) to afford the desired product as a colourless glassy solid (0.23 g, 0.50 mmol, 48%) m.p.: 89.9-91.2 °C;  $R_f$ : 0.50 (9:1 Chloroform-MeOH);  $\delta_H$  (500 MHz, DMSO-d<sub>6</sub>): 8.38-8.33 (3H, m, 6-HAr, 2'-HAr and 6'-HAr), 7.60 2H, brs, SNH<sub>2</sub>), 7.54-7.45 (3H, m, 3'-HAr, 4'-HAr and 5'-HAr), 7.42 2H, brs, NH<sub>2</sub>), 6.34 (1H, app s, 2-HFuryl), 5.36 1H, d, J 6.3, 3-HFuryl), 5.20 (1H, app s, 4-HFuryl), 4.46 (1H, app s, 5-HFuryl), 4.25-4.15 (2H, m, CH<sub>2</sub>), 1.60 (3H, s, CH<sub>3</sub>a), 1.38 (3H, s, CH<sub>3</sub>b);  $\delta_C$  (125 MHz, DMSO-d<sub>6</sub>): 158.6 (C2Ar), 156.4 (C4Ar), 150.3 (C8Ar), 140.8 (C6Ar), 138.7 (C1'Ar), 130.2 (C4'Ar), 129.2 (C3'Ar and C5'Ar), 128.7 (C6'Ar and C2'Ar), 119.1 (C9Ar), 89.3 (C2Furyl), 84.1 (C5Furyl), 83.8 (C3Furyl), 81.6 (C4Furyl), 68.6 (CH<sub>2</sub>), 27.5 (CH<sub>3</sub>a), 25.7 (CH<sub>3</sub>b);  $\nu_{max}$ /cm<sup>-1</sup> (solid): 3354, 2936, 1628, 1376; HPLC:  $T_r$  = 2.27 (100% rel. area);  $m/z$  (ES): (Found: [M+H]<sup>+</sup>, 463.1401. C<sub>19</sub>H<sub>22</sub>N<sub>6</sub>O<sub>6</sub>S requires [M+H], 463.1394.  $[\alpha]_D$  = 17.6° (c 0.1, MeOH).

**Preparation of [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-propenyl-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl sulfamate (19).** Preparation was *via* general method B using 2'3'-O-Isopropylidene-2-propenyladenosine (**12**) (0.15 g, 0.43 mmol) to afford the desired product as a pale yellow oil (0.14 g, 0.33 mmol, 77%)  $R_f$ : 0.47 (9:1 Chloroform-MeOH);  $\delta_H$  (500 MHz, DMSO-d<sub>6</sub>): 8.25 (1H, s, 6-HAr), 7.61 2H, brs, SNH<sub>2</sub>), 7.23 (2H, brs, NH<sub>2</sub>), 6.90 (1H, dd, J 13.4 and 6.6, 2-HPropyl), 6.36 (1H, d, J 13.4, 1-HPropyl), 6.24 (1H, app s, 2-HFuryl), 5.42 (1H, app s, 3-HFuryl), 5.15 (1H, app s, 4-HFuryl), 4.40 (1H, app s, 5-HFuryl), 4.35-4.25 (2H, m, CH<sub>2</sub>), 1.90 (3H, d, J 6.6, CH<sub>3</sub> Propyl), 1.57 (3H, s, CH<sub>3</sub>a), 1.36 (3H, s, CH<sub>3</sub>b);  $\delta_C$  (125 MHz, DMSO-d<sub>6</sub>): 171.0 (C2Ar), 156.1 (C4Ar), 150.9 (C8Ar), 140.0 (C6Ar), 125.5 (C2Pro), 119.4 (C9Ar), 117.7 (C1Pro), 112.4 (Acetyl C), 88.2 (C2Furyl), 83.4 (C5Furyl), 83.1 (C3Furyl), 81.4 (C4Furyl), 70.5 (CH<sub>2</sub>), 26.7 (CH<sub>3</sub>a), 25.0 (CH<sub>3</sub>b), 18.8 (CH<sub>3</sub>Pro);  $\nu_{max}$ /cm<sup>-1</sup> (solid): 3326, 3182, 2987, 2938, 1622, 1585, 1374; HPLC:  $T_r$  = 2.14 (67% rel. area);  $m/z$  (ES): (Found: [M+H]<sup>+</sup>, 427.1400. C<sub>16</sub>H<sub>22</sub>N<sub>6</sub>O<sub>6</sub>S requires [M+H], 427.1394.  $[\alpha]_D$  = 16.5° (c 0.1, MeOH).

**Preparation of [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-(furan-2-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl sulfamate (20).** Preparation was *via* general method B using 2'3'-O-Isopropylidene-2-(furan-2-yl)adenosine (**13**) (0.25g, 0.67 mmol) to afford the desired product as a colourless glassy solid (0.20 g, 0.45 mmol, 67%) m.p.: 83.3-85.2 °C;  $R_f$ : 0.46 (9:1 Chloroform-MeOH);  $\delta_H$  (500 MHz, DMSO-d<sub>6</sub>): 8.31 (1H, s, 6-HAr), 7.83 (1H, s, 3'-HAr), 7.56 (2H, brs, SNH<sub>2</sub>), 7.47 (2H, brs, NH<sub>2</sub>), 7.12 (1H, d, J 3.2, 5'-HAr), 6.65 (1H, dd, J 3.2 and 1.8, 4'-HAr), 6.30 (1H, d, J 1.9, 2-HFuryl), 5.44 (1H, d, J 6.2 3-HFuryl), 5.23 (1H, dd, J 6.2 and 3.5, 4-HFuryl), 4.43 (1H, app s, 5-HFuryl), 4.35-4.15 (2H, m, CH<sub>2</sub>), 1.58 (3H, s, CH<sub>3</sub>a), 1.36 (3H, s, CH<sub>3</sub>b);  $\delta_C$  (125 MHz, DMSO-d<sub>6</sub>): 159.5 (C2Ar), 155.8 (C4Ar), 152.3 (C1'Ar), 150.6 (C8Ar), 145.0 (C3'Ar), 140.0 (C6Ar), 119.4 (C9Ar), 114.4 (Acetyl C),

113.3 (C5'Ar), 112.1 (C4'Ar), 90.7 (C2Furyl), 84.4 (C5Furyl), 83.3 (C3Furyl), 81.1 (C4Furyl), 70.5 (CH<sub>2</sub>), 26.7 (CH<sub>3</sub>a), 24.5 (CH<sub>3</sub>b);  $\nu_{\max}$ / cm<sup>-1</sup> (solid): 3319, 3161, 2988, 1628, 1577, 1361; HPLC: T<sub>r</sub>= 2.27 (51% rel. area); *m/z* (ES): (Found: [M+H]<sup>+</sup>, 453.1189. C<sub>17</sub>H<sub>20</sub>N<sub>6</sub>O<sub>7</sub>S requires [M+H], 453.1187. [ $\alpha$ ]<sub>D</sub> = 1.4° (c 0.1, MeOH).

**Preperation of [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-(thiophen-3-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl sulfamate (21).** Preparation was *via* general method B using 2'3'-O-Isopropylidene-2-(thiophen-3-yl)adenosine (**14**) (0.45 g, 1.16 mmol) to afford the desired product as a pale brown oil (0.51 g, 1.09 mmol, 94%) *R*<sub>f</sub>: 0.48 (9:1 Chloroform-MeOH);  $\delta_{\text{H}}$  (500 MHz, DMSO-d<sub>6</sub>): 8.31 (1H, s, 6-HAr), 8.18 (1H, d, J 2.0, 2'-HAr), 7.77 (1H, dd, J 6.1 and 2.0, 5'-HAr), 7.58 (2H, brs, SNH<sub>2</sub>), 7.42 (1H, d, J 6.1, 4'-H), 7.38 (2H, brs, NH<sub>2</sub>), 6.31 (1H, d, J 2.2, 2-HFuryl), 5.54 (1H, d, J 4.0, 3-HFuryl), 5.20 (1H, d, J 4.0, 4-H Furyl), 4.44 (1H, app s, 5-HFuryl), 4.35-4.15 (2H, m, CH<sub>2</sub>), 1.60 (3H, s, CH<sub>3</sub>a), 1.37 (3H, s, CH<sub>3</sub>b);  $\delta_{\text{C}}$  (125 MHz, DMSO-d<sub>6</sub>): 169.5 (C2Ar), 155.9 (C4Ar), 149.4 (C8Ar), 142.2 (C6Ar), 134.8 (C4'Ar), 132.4 (C5'Ar), 126.4 (C9Ar), 125.0 (C2'Ar), 117.9 (C1'Ar), 113.5 (Acetyl C), 88.9 (C2Furyl), 83.7 (C5Furyl), 83.2 (C3Furyl), 81.2 (C4Furyl), 68.1 (CH<sub>2</sub>), 26.7 (CH<sub>3</sub>a), 25.1 (CH<sub>3</sub>b);  $\nu_{\max}$ / cm<sup>-1</sup> (solid): 3324, 3200, 2988, 1619, 1398; HPLC: T<sub>r</sub>= 2.23 (82% rel. area); *m/z* (ES): (Found: [M+H]<sup>+</sup>, 469.0958. C<sub>17</sub>H<sub>20</sub>N<sub>6</sub>O<sub>6</sub>S<sub>2</sub> requires [M+H], 469.0959. [ $\alpha$ ]<sub>D</sub> = 6.0° (c 0.1, MeOH).

**Preperation of [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-(pyridine-3-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl sulfamate (22).** Preparation was *via* general method B using 2'3'-O-Isopropylidene-2-(pyridine-3-yl)adenosine (**15**) (0.29 g, 0.75 mmol) to afford the desired product as a colourless glassy solid (0.25 g, 0.54 mmol, 72%) m.p.: 93.1-94.7 °C; *R*<sub>f</sub>: 0.39 (9:1 Chloroform-MeOH);  $\delta_{\text{H}}$  (500 MHz, DMSO-d<sub>6</sub>): 9.48 (1H, s, 2'-HAr), 8.67-8.59 (2H, m, 6'-H and 4'-H), 8.37 (1H, s, 6-HAr), 7.65-7.50 (5H, m, SNH<sub>2</sub>, NH<sub>2</sub> and 3'-HAr), 6.34 (1H, d, J 2.2, 2-HFuryl), 5.58 (1H, d, J 3.7 3-HFuryl), 5.19 (1H, dd, J 6.0 and 3.7, 4-HFuryl), 4.45 (1H, app s, 5-HFuryl), 4.30-4.20 (2H, m, CH<sub>2</sub>), 1.60 (3H, s, CH<sub>3</sub>a), 1.39 (3H, s, CH<sub>3</sub>b);  $\delta_{\text{C}}$  (125 MHz, DMSO-d<sub>6</sub>): 161.7 (C2Ar), 155.8 (C4Ar), 150.8 (C4'Ar), 150.6 (C8Ar), 149.5 (C2'Ar), 141.1 (C6Ar), 135.4 (C6'Ar), 133.2 (C1'Ar), 124.0 (C5'Ar), 119.1 (C9Ar), 114.2 (Acetyl C), 89.4 (C2Furyl), 84.0 (C5Furyl), 83.7 (C3Furyl), 81.6 (C4Furyl), 68.0 (CH<sub>2</sub>), 27.5 (CH<sub>3</sub>a), 25.7 (CH<sub>3</sub>b);  $\nu_{\max}$ / cm<sup>-1</sup> (solid): 3345, 3180, 2985, 1632, 1580, 1374; HPLC: T<sub>r</sub>= 2.04 (100% rel. area); *m/z* (ES): (Found: [M+H]<sup>+</sup>, 464.1357. C<sub>18</sub>H<sub>21</sub>N<sub>7</sub>O<sub>6</sub>S requires [M+H], 464.1347. [ $\alpha$ ]<sub>D</sub> = 53.5° (c 0.1, MeOH).

**Preparation of N-Boc-Ser(bzl)-OSu (23).** To a stirred solution of N-Boc-(Bzl)-Ser-OH (0.50 g, 1.69 mmol, 1.0 equiv.) in EtOAc/Dioxane (1:1, 10 mL) cooled to 0 °C were added N-hydroxysuccinimide (0.21 g, 1.78 mmol, 1.05 equiv.) and DCC (0.37 g, 1.78 mmol, 1.05 equiv.). The resulting mixture was stirred at room temperature overnight. The reaction mixture was filtered through Celite® and the filtrate concentrated

*in vacuo*. The residue was re-suspended in EtOAc (35 mL), washed with 5% NaHCO<sub>3</sub> (3 × 5 mL), water (2 × 10 mL), and brine (10 mL). The organic phase was dried (MgSO<sub>4</sub>) and concentrated in vacuo to afford the desired product as a colourless solid which was used without further purification. (0.35 g, 0.90 mmol, 53 %) m.p.: 98.3-99.2 °C; *R*<sub>f</sub>: 0.5 (9:1 DCM-MeOH); δ<sub>H</sub> (500 MHz, DMSO-d<sub>6</sub>): 7.45-7.28 (5H, m, H-Bzl), 4.69 (1H, d, J 5.7, H-Chiral), 4.55 (2H, s, CH<sub>2</sub>Bzl), 3.80 (2H, d, J 5.7, CH<sub>2</sub>), 2.82 (4H, brs, CH<sub>2</sub>C=O), 1.41 (9H, s, BOC); δ<sub>C</sub> (125 MHz, DMSO-d<sub>6</sub>): 169.8 (Succinimide C=O), 166.8 (C=O), 155.2 (BOC C=O), 137.8 (C1Ar), 128.2 (C3 and C5), 127.5 (C4), 127.5 (C2 and C6), 78.9 (C BOC), 72.3 (CH<sub>2</sub>Bzl), 68.6 (CH<sub>2</sub>C), 52.3 (Chiral C), 28.1 (CH<sub>3</sub> × 3), 25.4 (CH<sub>2</sub> × 2); ν<sub>max</sub>/ cm<sup>-1</sup> (solid): 3366, 2970, 1741, 1518, 1366; HPLC: T<sub>r</sub> = 2.61 (83% rel. area); *m/z* (ES): (Found: [M+H]<sup>+</sup>, 393.1663. C<sub>19</sub>H<sub>24</sub>N<sub>2</sub>O<sub>7</sub> requires [M+H], 393.1656. [α]<sub>D</sub> = -7.6° (c 0.1, MeOH).

**Preparation of tert-butyl N-[(2S)-1-[([(3aR, 4R, 6R, 6aR)-6-(6-amino-2-chloro-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methoxy)sulfonyl]amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (24).** Preparation was *via* general method C using [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-chloro-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl sulfamate (16) (0.15 g, 0.36 mmol) to afford the desired product as a colourless glassy solid (0.19 g, 0.46 mmol, 94%) m.p.: 168.6-170.2 °C; *R*<sub>f</sub>: 0.51 (9:1 DCM-MeOH); δ<sub>H</sub> (500 MHz, DMSO-d<sub>6</sub>): 8.43 (1H, s, 6-HAr), 7.83 (2H, brs, NH<sub>2</sub>), 7.30-7.20 (5H, m, Bzl), 6.10 (1H, d, J 5.7, 2-HFuryl), 6.04 (1H, brs, NH), 5.25 (1H, d, J 5.7, 3-HFuryl), 4.94 (1H, d, J 5.7, 4-HFuryl), 4.47 (2H, s, CH<sub>2</sub> Bzl), 4.35 (1H, d, J 5.7, 5-HFuryl), 4.07-4.01 (3H, m, CH<sub>2</sub>Chiral and Chiral H), 3.69-3.67 (2H, m, CH<sub>2</sub>), 1.55 (3H, s, CH<sub>3</sub>a), 1.38 (9H, s, CH<sub>3</sub> × 3), 1.29 (3H, s, CH<sub>3</sub>b); δ<sub>C</sub> (125 MHz, DMSO-d<sub>6</sub>): 171.6 (C=O), 157.8 (C4Ar), 155.4 (C=O BOC), 153.9 (C2Ar), 151.0 (C8Ar), 140.3 (C6Ar), 138.0 (C1Bzl), 128.7 (C3Bzl and C5Bzl), 128.3 (C2Bzl and C6Bzl), 127.9 (C4Bzl), 119.1 (C9Ar), 114.4 (C acetate), 90.6 (C2Furyl), 84.4 (C5Furyl), 83.3 (C3Furyl), 81.4 (C4Furyl), 79.5 (BOC C), 73.7 (CH<sub>2</sub> Bzl), 70.5 (CH<sub>2</sub>O), 69.8 (CH<sub>2</sub>C), 54.9 (Chiral C), 28.3 (CH<sub>3</sub> × 3), 26.7 (CH<sub>3</sub>a), 24.9 (CH<sub>3</sub>b); ν<sub>max</sub>/ cm<sup>-1</sup> (solid): 3330, 1691, 1637, 1304; HPLC: T<sub>r</sub> = 2.59 (100% rel. area); *m/z* (ES): (Found: [M+H]<sup>+</sup>, 698.2012. C<sub>28</sub>H<sub>36</sub>ClN<sub>7</sub>O<sub>10</sub>S requires [M+H], 698.2006. [α]<sub>D</sub> = -50.4° (c 0.1, MeOH).

**Preparation of tert-butyl N-[(2S)-1-[([(3aR, 4R, 6R, 6aR)-6-(6-amino-2-iodo-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methoxy)sulfonyl]amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (25).** Preparation was *via* general method C using [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-iodo-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl sulfamate (17) (0.20 g, 0.39 mmol) to afford the desired product as a colourless glassy solid (40.4 mg, 0.05 mmol, 13%) m.p.: 166.2-168.9 °C; *R*<sub>f</sub>: 0.54 (DCM-MeOH); δ<sub>H</sub> (400 MHz, DMSO-d<sub>6</sub>): 8.38 (1H, s, 6-HAr), 7.75 (2H, brs, NH<sub>2</sub>), 7.30-7.20 (5H, m, Bzl), 6.07 (1H, app s, 2-HFuryl), 5.24 (1H, app s, 3-HFuryl), 4.92 (1H, app s, 4-HFuryl), 4.42 (2H, app s, CH<sub>2</sub> Bzl), 4.35 (1H, app s, 5-HFuryl), 4.08-4.01 (3H, m, CH<sub>2</sub> Chiral and

Chiral H) 3.70-3.62 (2H, m, CH<sub>2</sub>), 1.54 (3H, s, CH<sub>3</sub>a), 1.38 (9H, s, CH<sub>3</sub> × 3), 1.20 (3H, s, CH<sub>3</sub>b); δ<sub>C</sub> (100 MHz, DMSO-d<sub>6</sub>): 174.5 (C=O), 157.3 (C<sub>4</sub>Ar), 155.5 (C=O BOC), 152.3 (C<sub>8</sub>Ar), 139.0 (C<sub>6</sub>Ar), 137.7 (C<sub>1</sub>Bzl), 128.7 (C<sub>5</sub>Bzl and C<sub>3</sub>Bzl), 128.1 (C<sub>4</sub>Bzl), 127.8 (C<sub>2</sub>'Ar and C<sub>6</sub>'Ar), 118.5 (C<sub>9</sub>Ar), 116.4 (C<sub>2</sub>Ar), 114.4 (Acetyl C), 88.3 (C<sub>2</sub>Furyl), 82.8 (C<sub>5</sub>Furyl), 81.5 (C<sub>3</sub>Furyl), 79.6 (BOC C), 79.0 (C<sub>4</sub>Furyl), 73.5 (CH<sub>2</sub> Bzl); 69.9 (CH<sub>2</sub>O), 67.4 (CH<sub>2</sub>C), 56.4 (Chiral C), 28.3 (CH<sub>3</sub> × 3), 26.4 (CH<sub>3</sub>a), 24.9 (CH<sub>3</sub>b); ν<sub>max</sub>/cm<sup>-1</sup> (solid): 3330, 1691, 1637, 1304; HPLC: T<sub>r</sub> = 2.67 (100% rel. area); m/z (ES): (Found: [M+Na]<sup>+</sup>, 812.1. C<sub>28</sub>H<sub>36</sub>N<sub>7</sub>O<sub>10</sub>S requires [M+Na], 812.1. [α]<sub>D</sub> = -52.2° (c 0.1, MeOH).

**Preparation of tert-butyl N-[(2S)-1-((((3aR, 4R, 6R, 6aR)-6-(6-amino-2-phenyl-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl)methoxy)sulfonyl)amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (26).** Preparation was *via* general method C using [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-phenyl-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl sulfamate (18) (0.20 g, 0.43 mmol) to afford the desired product as a colourless glassy solid (0.21 g, 0.29 mmol, 67%) m.p.: 155.8-157.7 °C; R<sub>f</sub>: 0.64 (9:1 DCM-MeOH); δ<sub>H</sub> (500 MHz, DMSO-d<sub>6</sub>): 8.42 (1H, s, 6-HAr), 8.37 (2H, d, J 6.9 6'-HAr and 2'-HAr), 7.57-7.41 (3H, m, 5'-HAr, 4'-HAr and 3'-HAr), 7.38 (2H, brs, NH<sub>2</sub>), 7.26-7.24 (5H, m, 2-HBzl, 3-HBzl, 4-HBzl, 5-HBzl and 6-HBzl), 6.27 (1H, d, J 3.0, 2-HFuryl), 6.06 (1H, d J 8.1, NH), 5.46 (1H, d, J 3.0, 3-HFuryl), 5.07 (1H, app s, 4-HFuryl), 4.45-4.41 (3H, m, BzlCH<sub>2</sub>, 5-HFuryl), 4.05 (2H, d, J 4.9, CH<sub>2</sub>O), 3.96-3.92 (1H, m, Chiral H), 3.67-3.58 (2H, m, Chiral CH<sub>2</sub>), 1.59 (3H, s, CH<sub>3</sub>a), 1.39 (9H, s, CH<sub>3</sub> × 3), 1.35 (3H, s, CH<sub>3</sub>b); δ<sub>C</sub> (125 MHz, DMSO-d<sub>6</sub>): 173.5 (C=ONH), 158.7 (C<sub>2</sub>Ar), 156.8 (C<sub>4</sub>Ar), 156.4 (C=O BOC), 150.6 (C<sub>8</sub>Ar), 140.0 (C<sub>6</sub>Ar), 138.5 (C<sub>1</sub>'Ar and C<sub>1</sub>Bzl), 130.4 (C<sub>4</sub>'Ar), 129.6-127.1 (C<sub>2</sub>Bzl, C<sub>3</sub>Bzl, C<sub>4</sub>Bzl, C<sub>5</sub>Bzl, C<sub>6</sub>Bzl, C<sub>2</sub>'Ar, C<sub>3</sub>'Ar, C<sub>5</sub>'Ar and C<sub>6</sub>'Ar), 118.0 (C<sub>9</sub>Ar), 112.5 (Acetyl C), 88.8 (C<sub>2</sub>Furyl), 83.5 (C<sub>5</sub>Furyl), 83.4 (C<sub>3</sub>Furyl), 81.6 (C<sub>4</sub>Furyl), 79.5 (BOC C), 71.8 (CH<sub>2</sub> Bzl), 71.1 (Chiral CH<sub>2</sub>), 67.1 (CH<sub>2</sub>), 57.1 (Chiral C), 28.2 (CH<sub>3</sub> × 3), 27.1 (CH<sub>3</sub>a), 25.2 (CH<sub>3</sub>b); ν<sub>max</sub>/cm<sup>-1</sup> (solid): 3358, 3193, 2980, 1629, 1575, 1438; HPLC: T<sub>r</sub> = 2.54 (100% rel. area); m/z (ES): (Found: [M+H]<sup>+</sup>, 740.2718. C<sub>34</sub>H<sub>41</sub>N<sub>7</sub>O<sub>10</sub>S requires [M+H], 740.2708. [α]<sub>D</sub> = 17.9° (c 0.1, MeOH).

**Preparation of tert-butyl N-[(2S)-1-((((3aR, 4R, 6R, 6aR)-6-(6-amino-2-propenyl-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl)methoxy)sulfonyl)amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (27).** Preparation was *via* general method C using [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-propenyl-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl

sulfamate (**19**) (0.14 g, 0.33 mmol) to afford the desired product as a colourless glassy solid (64.9 mg, 0.09 mmol, 28%) m.p.: 142.9-144.3 °C;  $R_f$ : 0.47 (9:1 DCM-MeOH);  $\delta_H$  (500 MHz, DMSO- $d_6$ ): 8.33 (1H, s, 6-HAr), 7.30-7.26 (5H, m, 2'-HAr, 3'-HAr, 4'-HAr, 5'-HAr and 6'-HAr), 7.18 (2H, brs, NH<sub>2</sub>), 6.92 (1H, dd, J 15.3 and 7.0, 2-HPro), 6.35 (1H, d, J 15.3, 1-HPro), 6.15 (1H, d, J 2.8, 2-HFuryl), 6.06 (1H, d, J 7.7 NH), 5.33 (1H, dd, J 6.3 and 2.8, 3-HFuryl), 5.04 (1H, app t, J 6.3, 4-HFuryl), 4.42 (2H, d, J 9.9, CH<sub>2</sub> Bzl), 4.36 (1H, d J 2.4, 5-HFuryl), 4.15-3.95 (3H, m, CH<sub>2</sub>O and ChiralH), 3.73-3.54 (2H, m, CH<sub>2</sub> Chiral), 1.89 (3H, dd, J 7.0 and 1.5, Me), 1.56 (3H, s, CH<sub>3</sub>a), 1.39 (9H, s, CH<sub>3</sub> × 3), 1.33 (3H, s, CH<sub>3</sub>b);  $\delta_C$  (125 MHz, DMSO- $d_6$ ): 172.5 (C2Ar), 170.1 (C=ONH), 156.1 (C4Ar), 155.4 (C=OBOC), 150.8 (C8Ar), 140.3 (C6Ar), 137.8 (C1'Ar), 128.7-127.5 (Aromatics), 125.2 (C2pro), 119.1 (C9Ar), 117.7 (C1Pro), 114.3 (Acetyl C), 88.9 (C2Furyl), 83.7 (C5Furyl), 83.4 (C3Furyl), 81.7 (C4Furyl), 79.5 (BOC C), 71.8 (CH<sub>2</sub> Bzl), 71.1 (CH<sub>2</sub>O), 67.1 (CH<sub>2</sub> Chiral), 56.5 (Chiral C), 28.2 (CH<sub>3</sub> × 3), 27.1 (CH<sub>3</sub>a), 25.2 (CH<sub>3</sub>b), 17.8 (CH<sub>3</sub>Pro);  $\nu_{max}/\text{cm}^{-1}$  (solid): 3359, 2968, 1627, 1579, 1345; HPLC:  $T_r$  = 2.45 (78% rel. area);  $m/z$  (ES): (Found:  $[M+H]^+$ , 704.2719. C<sub>31</sub>H<sub>41</sub>N<sub>7</sub>O<sub>10</sub>S requires  $[M+H]$ , 704.2708.  $[\alpha]_D$  = -8.6° (c 0.1, MeOH).

**Preparation of tert-butyl N-[(2S)-1-((((3aR, 4R, 6R, 6aR)-6-(6-amino-2-(furan-2-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl)methoxy)sulfonyl)amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (**28**).** Preparation was *via* general method C using [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-(furan-2-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl]methyl sulfamate (**20**) (0.20 g, 0.44 mmol) to afford the desired product as a colourless glassy solid (0.10 g, 0.14 mmol, 32%) m.p.: 157.7-159.4 °C;  $R_f$ : 0.50 (9:1 DCM-MeOH);  $\delta_H$  (500 MHz, DMSO- $d_6$ ): 8.40 (1H, s, 6-HAr), 7.81 (1h, s, 3'-HAr), 7.42 (2H, brs, NH<sub>2</sub>), 7.32-7.28 (5H, m, Bzl), 7.24 (1H, d, J 4.1, 5'-HAr), 6.62 (1H, dd, J 4.1 and 1.87, 4'-HAr), 6.21 (1H, d, J 2.8, 2-HFuryl), 6.06 (1H, app s, NH), 5.36 (1H, app s, 3-HFuryl), 5.08 (1H, app s, 4-HFuryl), 4.48-4.32 (3H, m, CH<sub>2</sub> Bzl and 5-HFuryl), 4.15-3.90 (3H, m, CH<sub>2</sub>O and Chiral H), 3.70-3.50 (2H, m, CH<sub>2</sub>Chiral), 1.57 (3H, s, CH<sub>3</sub>a), 1.38 (9H, s, CH<sub>3</sub> × 3), 1.33 (3H, s, CH<sub>3</sub>b);  $\delta_C$  (125 MHz, DMSO- $d_6$ ): 170.0 (C=ONH), 158.3 (C2Ar), 156.4 (C4Ar), 155.3 (C=OBOC), 152.5 (C1Furan), 150.6 (C8Ar), 144.7 (C3Furan), 140.2 (C6Ar), 137.7 (C1Bzl), 128.8-127.7 (Aromatics), 119.3 (C9Ar), 114.2 (Acetyl C), 112.4 (C5Furan), 111.9 (C4Furan), 89.2 (C2Furyl), 84.4 (C5Furyl), 84.1 (C3Furyl), 82.1 (C4Furyl), 79.6 (BOC C), 72.2 (CH<sub>2</sub> Bzl), 71.0 (CH<sub>2</sub>O), 67.7 (CH<sub>2</sub> Chiral), 56.9 (Chiral C), 28.7 (CH<sub>3</sub> × 3), 27.6 (CH<sub>3</sub>a), 25.7 (CH<sub>3</sub>b);  $\nu_{max}/\text{cm}^{-1}$  (solid): 3342, 1674, 1572, 1364; HPLC:  $T_r$  = 2.51 (73% rel. area);  $m/z$  (ES): (Found:  $[M+H]^+$ , 730.2513. C<sub>32</sub>H<sub>39</sub>N<sub>7</sub>O<sub>11</sub>S requires  $[M+H]$ , 730.2501.  $[\alpha]_D$  = -24.8° (c 0.1, MeOH).

**Preparation of tert-butyl N-[(2S)-1-((((3aR, 4R, 6R, 6aR)-6-(6-amino-2-(thiophen-3-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl)methoxy)sulfonyl)amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (**29**).** Preparation was *via* general method C using [(3aR, 4R, 6R, 6aR)-6-

(6-amino-2-(thiophen-3-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl)methyl sulfamate (**21**) (0.50 g, 1.06 mmol) to afford the desired product as a colourless glassy solid (0.33 g, 0.45 mmol, 42%) m.p.: 156.2-158.0 °C;  $R_f$ : 0.58 (9:1 DCM-MeOH);  $\delta_H$  (500 MHz, DMSO-d<sub>6</sub>): 8.38 (1H, s, 6-HAr), 8.19 (1H, dd, J 3.1 and 1.1, 2'-HAr), 7.78 (1H, dd, J 5.1 and 1.1 4'-HAr), 7.57 (1H, dd, J 5.0 and 3.1, 5'-HAr), 7.30 (2H, brs, NH<sub>2</sub>), 7.29-7.25 (5H, m, bz1), 6.23 (1H, d, J 3.0, 2-HFuryl), 6.07 (1H, app s, NH), 5.41 (1H, d, J 2.8, 3-HFuryl), 5.07 (1H, d, J 2.8, 4-HFuryl), 4.41 (3H, app d, J 9.9, CH<sub>2</sub>Bzl) and 5-HFuryl, 4.10-4.00 (2H, m, CH<sub>2</sub>O), 3.98-3.94 (1H, m, Chiral H), 3.70-3.50 (2H, m, CH<sub>2</sub>Chiral), 1.58 (3H, s, CH<sub>3</sub>a), 1.39 (9H, s, CH<sub>3</sub> × 3), 1.35 (3H, s, CH<sub>3</sub>b);  $\delta_C$  (125 MHz, DMSO-d<sub>6</sub>): 173.2 (C2Ar), 170.1 (C=ONH), 156.1 (C4Ar), 155.9 (C=OBoc), 150.8 (C8Ar), 142.8 (C6Ar), 138.5 (C1Bzl), 128.0 (C5Bzl and C3Bzl), 127.3 (C6Bzl and C2Bzl), 127.1 (C4Bzl), 126.1 (C4Thio), 125.1 (C5Thio), 120.0 (C2Thio), 119.4 (C9Ar), 117.0 (C1Thio), 113.1 (Acetyl C), 88.7 (C2Furyl), 83.6 (C5Furyl), 83.3 (C3Furyl), 81.6 (C4Furyl), 79.7 (Boc C), 73.4 (CH<sub>2</sub> Bzl), 71.7 (CH<sub>2</sub>O), 68.9 (CH<sub>2</sub> Chiral), 56.2 (Chiral C), 28.2 (CH<sub>3</sub> × 3), 27.1 (CH<sub>3</sub>a), 25.2 (CH<sub>3</sub>b);  $\nu_{max}$ /cm<sup>-1</sup> (solid): 3250, 3052, 1675, 1526, 1374; HPLC:  $T_r$  = 2.53 (100% rel. area);  $m/z$  (ES): (Found:  $[M+H]^+$ , 746.2286. C<sub>32</sub>H<sub>39</sub>N<sub>7</sub>O<sub>10</sub>S<sub>2</sub> requires  $[M+H]$ , 746.2273.  $[\alpha]_D$  = 28.9° (c 0.1, MeOH).

**Preparation of tert-butyl N-[(2S)-1-(((3aR, 4R, 6R, 6aR)-6-(6-amino-2-(pyridine-3-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl)methoxy)<sup>2</sup>sulfonyl)amino]-3-(benzyloxy)-1-oxopropan-2-yl]carbamate (**30**).** Preparation was *via* general method C using [(3aR, 4R, 6R, 6aR)-6-(6-amino-2-(pyridine-3-yl)-9H-purin-9-yl)-2,2-dimethyl-tetrahydro-2H-furo[3,4-d][1,3]dioxol-4-yl)methyl sulfamate (**22**) (0.25 g, 0.54 mmol) to afford the desired product as a colourless glassy solid (0.12 g, 0.16 mmol, 29%) m.p.: 167.2-164.5 °C;  $R_f$ : 0.39 (9:1 DCM-MeOH);  $\delta_H$  (500 MHz, DMSO-d<sub>6</sub>): 9.49 (1H, s, 2'-HAr), 8.64 (2H, d, J 5.7, 6'-HAr and 4'-HAr), 8.46 (1H, s, 6-HAr), 7.52 (3H, brs, NH<sub>2</sub> and 5'-HAr), 7.27-7.24 (5H, m, Bzl), 6.27 (1H, d, J 3.0, 2-HFuryl), 6.08 (1H, d, J 7.8, NH), 5.43 (1H, d, J 2.7, 3-HFuryl), 5.07 (1H, app s, 4-Hfuryl), 4.41 (3H, app s, CH<sub>2</sub> Bzl and 5-HFuryl), 4.05 (2H, d, J 4.6, CH<sub>2</sub>O), 3.94 (1H, s, Chiral H), 3.62 (2H, d J 6.6, CH<sub>2</sub> Chiral), 1.59 (3H, s, CH<sub>3</sub>a), 1.36 (9H, s, CH<sub>3</sub> × 3), 1.35 (3H, s, CH<sub>3</sub>b);  $\delta_C$  (125 MHz, DMSO-d<sub>6</sub>): 173.7 (C=ONH), 160.5 (C2Ar), 156.1 (C4Ar), 155.4 (C=OBoc), 150.2 (C4Pip), 150.1 (C8Ar), 148.9 (C2Pip), 140.1 (C6Ar), 137.7 (C1Bzl), 134.9 (C6Pip), 133.2 (C1Pip), 128.0 (C5Bzl and C3Bzl), 127.3 (C6Bzl and C2Bzl), 127.1 (C4Bzl), 123.5 (C5Pip), 119.4 (C9Ar), 114.2 (Acetyl C), 89.0 (C2Furyl), 83.5 (C5Furyl), 83.4 (C3Furyl), 81.5 (C4Furyl), 79.6 (Boc C), 73.7 (CH<sub>2</sub> Bzl), 71.7 (CH<sub>2</sub>O), 68.2 (CH<sub>2</sub> Chiral), 54.7 (Chiral C), 28.2 (CH<sub>3</sub> × 3), 27.1 (CH<sub>3</sub>a), 25.2 (CH<sub>3</sub>b);  $\nu_{max}$ /cm<sup>-1</sup> (solid): 3377, 2976, 1662, 1573, 1369; HPLC:  $T_r$  = 2.37 (100% rel. area);  $m/z$  (ES): (Found:  $[M+H]^+$ , 741.2674. C<sub>33</sub>H<sub>40</sub>N<sub>8</sub>O<sub>10</sub>S requires  $[M+H]$ , 741.2661.  $[\alpha]_D$  = -13.6° (c 0.1, MeOH).



**Protein expression and purification.** *EcSerRS* (from *E. coli* strain B ER2560) and *SaSerRS* (from *S. aureus* seg50 (1150)) were cloned into pET52b(+) vector (Merck Millipore, Germany) using the NcoI and SacI restriction sites allowing for the production of protein with a thrombin cleavable C-terminal His<sub>10</sub>-tag. *EcSerRS* and *SaSerRS* were overexpressed in Lemo21(DE3) cells grown in Auto Induction Media – Terrific Broth (Formedium) supplemented with 100 µg/ml ampicillin at 37°C for 8 hours followed by overnight growth at 25°C. Cells were harvested by centrifugation at 5000 rpm in a JLA 8.1000 rotor (Beckman Coulter) for 15 min, and the pellet was re-suspended in buffer A (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 30 mM Imidazole). The cells were disrupted by sonication at 70 % amplitude for 30 sec on ice and 8 pulses. The lysate was centrifuged at 18,000 rpm in a JA 25.50 rotor (Beckman Coulter) for 30 mins. The supernatant was decanted, passed through a 0.2 µm filter and applied to a 5 ml HisTrap column (GE healthcare, USA). The bound protein was eluted with a gradient of buffer B (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 500 mM Imidazole) (0-100% over 50 ml) on an ÄKTA Pure (GE healthcare, USA) at 2 ml/min. The protein was dialyzed into 2 L of buffer A with thrombin cleavage (1 unit/µg). The protein was passed through the 5 ml HisTrap column to remove the cleaved His-tag and other contaminants. The proteins typically present over 95 % purity at this stage as judged via SDS-PAGE gel and were taken for crystallization trials after dialysis into 20 mM Tris-HCl pH 7.5, 200 mM NaCl and 1 mM MgCl<sub>2</sub>. Further purification was used for protein used for kinetic and binding studies to ensure complete removal of thrombin using a HiLoad 16/600 Superdex 200 pg column (GE Healthcare, USA) in 20 mM Tris-HCl pH 7.5, 200 mM NaCl and 1 mM MgCl<sub>2</sub>. The purified protein was subsequently stored in 50 % glycerol at -80°C. *HsSerRS* was expressed and purified as previously described.<sup>35</sup>

### Crystallisation and structure solution.

Co-crystals of *EcSerRS* in the presence of SerSA were obtained from a drop set up in 96-well sitting drop format with 20 mg ml<sup>-1</sup> protein and ten-fold molar excess of SerSA. Drops consisted of 100 nl protein preincubated with SerSA and 100 nl reservoir solution with a reservoir volume of 95 µl. Crystals were obtained from a drop containing 0.2 M sodium phosphate monobasic monohydrate, pH 4.7 and 20 % (w/v) PEG 3350 following incubation at 4°C and cryoprotected in reservoir solution containing 25 % (v/v) ethylene glycol.

Co-crystals of His-tagged *SaSerRS* were obtained from a drop set up with 20 mg ml<sup>-1</sup> protein in the presence of ten-fold molar excess of SerSA in 24-well hanging drop format. Drops consisted of 1 µl protein preincubated with SerSA and 1 µl reservoir solution with a reservoir volume of 500 µl. Plates were incubated at 4°C and crystals obtained in 0.2 M sodium malonate pH 5.0 and 13 % (w/v) PEG 3350. Crystals were cryoprotected for 10 s in reservoir solution containing 20 % (v/v) ethylene glycol and ten-fold molar excess of SerSA.

Crystals of apo-*Ec*SerRS were obtained at 21°C from a 24-well hanging drop format as described above with 30 mg ml<sup>-1</sup> protein in a crystallisation condition consisting of 0.1 M sodium citrate pH 5.5, 0.8 M lithium sulfate and 0.05 M ammonium sulfate. A single crystal was soaked for 30 mins in 0.1 M sodium citrate pH 5.5, 0.75 M lithium sulfate, 0.05 M ammonium sulfate, 20 % (v/v) ethylene glycol and 100 mM compound **8** (10 % (v/v) DMSO in final solution).

All crystals were flash frozen in liquid nitrogen and diffraction data collected at 100 K at beamlines I03 and I04 (Diamond Light Source, United Kingdom). Data was indexed and integrated using iMosflm<sup>36</sup> and scaled using Aimless in CCP4<sup>37</sup> or autoPROC<sup>38</sup> was used in the DLS auto-processing pipeline. The crystal structure of aq\_298 (PDB 2DQ3, unpublished) was used as a search model in Phaser MR<sup>39</sup> to solve the structures of *Ec*SerRS and *Sa*SerRS by molecular replacement. Phenix<sup>40</sup> and Buster<sup>41</sup> were used for iterative rounds of refinement with model building carried out in COOT.<sup>42</sup> Figures were made using PyMOL (Schrödinger, LLC).

**Kinetic analyses.** SerRS assays were performed at 37°C in a Cary 100 UV/Vis double beam spectrophotometer with a thermostatted 6X6 cell changer. The final assay volume was 0.2 ml, containing 50 mM HEPES adjusted to pH 7.6, 10 mM MgCl<sub>2</sub>, 50 mM KCl, 1 mM dithiothreitol, 10% (v/v) dimethylsulphoxide, 10 mM D-glucose, 0.5 mM NADP<sup>+</sup>, 1.7 mM.min yeast hexokinase and 0.85 mM.min *L. mesenteroides* glucose 6-phosphate dehydrogenase (Roche, Germany). Concentrations of SerRS, amino acid, substrate (Ap4A, Sigma-Aldrich, Dorset, UK ) and pyrophosphate were as stated in the text (**Supplementary Table 3 and Supplementary Table 4**). Unless otherwise stated, background rates were acquired in the absence of amino acid, which was then added to initiate the full reaction. Assays were continuously monitored at 340 nm, to detect reduction of NADP<sup>+</sup> to NADPH, where  $\Delta\text{NADPH}; 340\text{nm} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$ . Kinetic constants relating to substrate dependencies and IC<sub>50</sub> values for inhibitors were extracted by non-linear regression using GraphPad Prism 7.00.

**Isothermal titration calorimetry.** Calorimetric titrations of *Ec*SerRS with SerSA and/or compound **8** were performed on a VP-ITC microcalorimeter (MicroCal) at 25°C and measured in triplicates. The gel-filtration purified *Ec*SerRS was concentrated and dialysed overnight against the ITC buffer (20 mM Tris-HCl, pH 7.5 and 200 mM NaCl) at 4°C. All the solutions were degassed by sonication. The overnight dialysis ITC buffer was used to prepare SerSA and compound **8** solutions. The *Ec*SerRS (3 µM for SerSA and 7 µM for compound **8**) in the sample cell (1.445 ml) was titrated with ligand solution (70 µM of SerSA and 140 µM of compound **8**) in the syringe (280 µl). The *Ec*SerRS - SerSA ITC experiments consisted of a preliminary 2 µl injection followed by 52 successive 5 µl injections. The *Ec*SerRS - compound **8** ITC experiments consisted of a preliminary 2 µl injection followed by 26 successive 10 µl injections. Each injection lasted

20 s with an interval of 120 s between consecutive injections. The solution in the reaction cell was stirred at 307 rpm throughout the experiments. The heat response data for the preliminary injection was discarded and the rest of the data was used to generate binding isotherm. The data were fit using either the one binding site model or the two independent binding sites model included in the Origin 7.0 (MicroCal). Thermodynamic parameters, including association constant ( $K_a$ ), enthalpy ( $\Delta H$ ), entropy ( $\Delta S$ ) and binding stoichiometry ( $N$ ) were calculated by iterative curve fitting of the binding isotherms. The Gibbs free energy was calculated using  $\Delta G = \Delta H - T\Delta S$ .

**Analytical ultracentrifugation.** All experiments were performed at 50000 rpm, using a Beckman Optima analytical ultracentrifuge with an An-50Ti rotor. Data were recorded using the absorbance (at 280 nm with 10  $\mu$ m resolution and recording scans every 20 seconds) and interference (recording scans every 60 seconds) optical detection systems. The density and viscosity of the buffer was measured experimentally using a DMA 5000M densitometer equipped with a Lovis 200ME viscometer module. The partial specific volume for the protein constructs were calculated using Sednterp from the amino acid sequences. For characterisation of the protein samples, SV scans were recorded for a dilution series, starting from 0.8 mg/mL. Where a ligand was included, this was present at 400  $\mu$ M (a 20-fold excess over the highest concentration protein sample). Data were processed using SEDFIT, fitting to the  $c(s)$  model.<sup>43</sup> Figures were made using GUSI.<sup>44</sup>

**Microbiological susceptibility testing.** Bacterial strains: *E. Coli* ATCC 25922 and *S. aureus* ATCC 25923 were used. MIC values were determined by broth micro-dilution method, in triplicate, using cation-adjusted Mueller Hinton broth (Sigma) according to the Clinical Laboratory Standards Institute (CLSI) guidelines<sup>28</sup>. Experiments were carried out in 96 well micro-titre plates (Star Labs) containing the medium plus Inhibitors 1-8 as appropriate. Inhibitors 1-8 were dissolved in water to a concentration of 256 $\mu$ g/ml and diluted in broth. Plates were incubated overnight at 37 °C for 18–20 h and MIC was determined by visual inspection. Additional data that support the findings of this study are available from the corresponding author upon reasonable request.

## Associated Content

### Supporting Information.

The following files are available free of charge.

Crystallographic refinement data, Structural overlay statistics, *in silico* docking scores and analysis, biological assays and determination of IC<sub>50</sub> values, ITC and AUC data, and Characterisation of target compounds (PDF)

## Accession Codes

The crystallographic data that support the findings of this study are available from the Protein Data Bank (<http://www.rcsb.org>). *Ec*SerRS:SerSA, 6R1M; *Sa*SerRS:SerSA, 6R1N; *Ec*SerRS:compound **8**, 6R1O.

Authors will release the atomic coordinates upon article publication.

## Author Information

### Corresponding Author

\* David I Roper Phone: +44 (0) 2476 528369 E-mail: david.roper@warwick.ac.uk

### Present Addresses

<sup>†</sup>Present address – The Institute of Cancer Research, 15 Cotswold Road, Sutton, SM2 5NG, United Kingdom

<sup>††</sup> Present address - Drug Discovery Unit, School of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom.

### Author Contribution

<sup>†</sup>R.C. and R.S. contributed equally to this work.

### Notes

The authors declare no competing financial interest.

### Acknowledgements

We acknowledge funding from Medical Research Council (MRC) for Innovation Grant MR/M017893/1 and BBSRC Flexible Talent Mobility Account award BB/R506588/1 that supported this work directly and EPSRC AMR bridging the gaps grant EP/M027503/1 that provided initial pump priming activity. We thank Diamond Light Source for beam time (proposal MX14692) and staff at beamlines I03 and I04 for their assistance. We would like to acknowledge Dr Michael Haertlein of the Institut Laue Langevin, Grenoble, France for supply of the *Hs*SerRS expression construct used in this study and Dr Yann-Vai Le Bihan of the Institute of Cancer Research, London, United Kingdom for advice and support during the structure refinement process. We acknowledge undergraduate students Archana Shah and Sarah Brocklesby for initial cloning of the bacterial synthetase genes.

### Abbreviations

SerRS, Seryl aminoacyl-tRNA synthetase. SerSA, Seryl sulfamoyl adenosine. AlaSA, alanyl sulfoamoyl adenosine. ThrSA, Threonyl sulfamoyl adenosine.

## References

1. Lenhard, B.; Orellana, O.; Ibba, M.; Weygand-Durasevic, I., tRNA Recognition and Evolution of Determinants in Seryl-tRNA Synthesis. *Nucleic Acids Res.* **1999**, *27* (3), 721-729.
2. F C Neidhardt; J Parker, a.; McKeever, W. G., Function and Regulation of Aminoacyl-tRNA Synthetases in Prokaryotic and Eukaryotic Cells. *Annual Review of Microbiology* **1975**, *29* (1), 215-250.
3. Schimmel, P.; Tao, J.; Hill, J., Aminoacyl tRNA Synthetases as Targets for New Anti-infectives. *FASEB J.* **1998**, *12* (15), 1599-1609.
4. Ho, J. M.; Bakkalbasi, E.; Soll, D.; Miller, C. A., Drugging tRNA Aminoacylation. *RNA Biol.* **2018**, *15* (4-5), 667-677.
5. Ataide, S. F.; Ibba, M., Small Molecules: Big Players in the Evolution of Protein Synthesis. *ACS Chem. Biol.* **2006**, *1* (5), 285-297.
6. Perona, J. J.; Gruic-Sovulj, I., Synthetic and Editing Mechanisms of Aminoacyl-tRNA Synthetases. *Top. Curr. Chem.* **2014**, *344*, 1-41.
7. Lloyd, A. J.; Potter, N. J.; Fishwick, C. W. G.; Roper, D. I.; Dowson, C. G., Adenosine Tetraphosphoadenosine Drives a Continuous ATP-Release Assay for Aminoacyl-tRNA Synthetases and Other Adenylate-Forming Enzymes. *ACS Chem. Biol.* **2013**, *8* (10), 2157-2163.
8. Cvetic, N.; Gruic-Sovulj, I., Synthetic and Editing Reactions of Aminoacyl-tRNA Synthetases using Cognate and Non-cognate Amino Acid Substrates. *Methods* **2017**, *113*, 13-26.
9. Dewan, V.; Reader, J.; Forsyth, K. M., Role of Aminoacyl-tRNA Synthetases in Infectious Diseases and Targets for Therapeutic Development. *Top. Curr. Chem.* **2014**, *344*, 293-329.
10. Van de Vijver, P.; Vondenhoff, G. H. M.; Denivelle, S.; Rozenski, J.; Verhaegen, J.; Van Aerschot, A.; Herdewijn, P., Antibacterial 5'-O-(N-dipeptidyl)-sulfamoyladenines. *Biorg. Med. Chem.* **2009**, *17* (1), 260-269.
11. Silvan, L. F.; Wang, J.; Steitz, T. A., Insights into Editing from an Ile-tRNA Synthetase Structure with tRNA<sup>Ile</sup> and Mupirocin. *Science* **1999**, *285* (5430), 1074-1077.
12. Guo, M.; Chong, Y. E.; Shapiro, R.; Beebe, K.; Yang, X. L.; Schimmel, P., Paradox of Mistranslation of Serine for Alanine caused by AlaRS Recognition Dilemma. *Nature* **2009**, *462* (7274), 808-812.
13. Belrhali, H.; Yaremchuk, A.; Tukalo, M.; Larsen, K.; Berthet-Colominas, C.; Leberman, R.; Beijer, B.; Sproat, B.; Als-Nielsen, J.; Grubel, G.; Legrand, J. F.; Lehmann, M.; Cusack, S., Crystal Structures at 2.5 Angstrom Resolution of Seryl-tRNA Synthetase Complexed with two Analogs of Seryl Adenylate. *Science* **1994**, *263* (5152), 1432-1436.
14. Fujinaga, M.; Berthet-Colominas, C.; Yaremchuk, A. D.; Tukalo, M. A.; Cusack, S., Refined Crystal Structure of the Seryl-tRNA Synthetase from *Thermus Thermophilus* at 2.5 Å Resolution. *J. Mol. Biol.* **1993**, *234* (1), 222-233.
15. Bilokapic, S.; Maier, T.; Ahel, D.; Gruic-Sovulj, I.; Soll, D.; Weygand-Durasevic, I.; Ban, N., Structure of the Unusual Seryl-tRNA Synthetase Reveals a Distinct Zinc-dependent Mode of Substrate Recognition. *EMBO J.* **2006**, *25* (11), 2498-2509.
16. Itoh, Y.; Sekine, S.; Kuroishi, C.; Terada, T.; Shirouzu, M.; Kuramitsu, S.; Yokoyama, S., Crystallographic and Mutational Studies of Seryl-tRNA Synthetase from the Archaeon *Pyrococcus Horikoshii*. *RNA Biol.* **2008**, *5* (3), 169-177.
17. Rocha, R.; Pereira, P. J.; Santos, M. A.; Macedo-Ribeiro, S., Unveiling the Structural Basis for Translational Ambiguity Tolerance in a Human Fungal Pathogen. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (34), 14091-14096.
18. Kekez, M.; Zanki, V.; Kekez, I.; Baranasic, J.; Hodnik, V.; Duchene, A. M.; Anderluh, G.; Gruic-Sovulj, I.; Matkovic-Calogovic, D.; Weygand-Durasevic, I.; Rokov-Plavec, J., Arabidopsis Seryl-tRNA Synthetase: the First Crystal Structure and Novel Protein Interactor of Plant Aminoacyl-tRNA Synthetase. *FEBS J.* **2019**, *286* (3), 536-554.

19. Itoh, Y.; Sekine, S.; Suetsugu, S.; Yokoyama, S., Tertiary Structure of Bacterial Selenocysteine tRNA. *Nucleic Acids Res.* **2013**, *41* (13), 6729-6738.
20. Xu, X.; Shi, Y.; Yang, X. L., Crystal Structure of Human Seryl-tRNA Synthetase and Ser-SA Complex Reveals a Molecular Lever Specific to Higher Eukaryotes. *Structure* **2013**, *21* (11), 2078-2086.
21. Chimnaronk, S.; Gravers Jeppesen, M.; Suzuki, T.; Nyborg, J.; Watanabe, K., Dual-mode Recognition of Noncanonical tRNAs(Ser) by Seryl-tRNA Synthetase in Mammalian Mitochondria. *EMBO J.* **2005**, *24* (19), 3369-3379.
22. Cusack, S.; Berthet-Colominas, C.; Hartlein, M.; Nassar, N.; Leberman, R., A Second Class of Synthetase Structure Revealed by X-ray Analysis of Escherichia Coli Seryl-tRNA Synthetase at 2.5 Å. *Nature* **1990**, *347* (6290), 249-255.
23. Zhang, B.; De Graef, S.; Nautiyal, M.; Pang, L.; Gadakh, B.; Froeyen, M.; Van Mellaert, L.; Strelkov, S. V.; Weeks, S. D.; Van Aerschot, A., Family-wide Analysis of Aminoacyl-sulfamoyl-3-deazaadenosine Analogues as Inhibitors of Aminoacyl-tRNA Synthetases. *Eur. J. Med. Chem.* **2018**, *148*, 384-396.
24. Schrödinger Release 2016-1: Maestro, version 10.6, Schrödinger, LLC, New York, NY, 2016.
25. Miyaura, N.; Yamada, K.; Suzuki, A., A New Stereospecific Cross-coupling by the Palladium-catalyzed Reaction of 1-alkenylboranes with 1-alkenyl or 1-alkynyl halides. *Tetrahedron Lett.* **1979**, *20* (36), 3437-3440.
26. Srikrishna, A.; Viswajanani, R.; Sattigeri, J. A.; Vijaykumar, D., A new, convenient reductive procedure for the deprotection of 4-methoxybenzyl (MPM) ethers to alcohols. *The Journal of Organic Chemistry* **1995**, *60* (18), 5961-5962.
27. Cusack, S.; Hartlein, M.; Leberman, R., Sequence, Structural and Evolutionary Relationships between Class 2 Aminoacyl-tRNA Synthetases. *Nucleic Acids Res.* **1991**, *19* (13), 3489-3498.
28. Methods for Dilution Antimicrobial Susceptibility Testing for Bacteria that Grow Aerobically, Approved Standard - eighth edition. CLSI. **2009**.
29. Richter, M. F.; Drown, B. S.; Riley, A. P.; Garcia, A.; Shirai, T.; Svec, R. L.; Hergenrother, P. J., Predictive Compound Accumulation Rules Yield a Broad-spectrum Antibiotic. *Nature* **2017**, *545*, 299.
30. Wu, Y.; Yu, K.; Xu, B.; Chen, L.; Chen, X.; Mao, J.; Danchin, A.; Shen, X.; Qu, D.; Jiang, H., Potent and Selective Inhibitors of Staphylococcus Epidermidis Tryptophanyl-tRNA Synthetase. *J. Antimicrob. Chemother.* **2007**, *60* (3), 502-509.
31. Kim, S. Y.; Lee, Y.-S.; Kang, T.; Kim, S.; Lee, J., Pharmacophore-based Virtual Screening: The Discovery of Novel Methionyl-tRNA Synthetase Inhibitors. *Bioorg. Med. Chem. Lett.* **2006**, *16* (18), 4898-4907.
32. Brown, M. J. B.; Mensah, L. M.; Doyle, M. L.; Broom, N. J. P.; Osbourne, N.; Forrest, A. K.; Richardson, C. M.; O'Hanlon, P. J.; Pope, A. J., Rational Design of Femtomolar Inhibitors of Isoleucyl tRNA Synthetase from a Binding Model for Pseudomonas Acid-A. *Biochemistry* **2000**, *39* (20), 6003-6011.
33. Teng, M.; Hilgers, M. T.; Cunningham, M. L.; Borchardt, A.; Locke, J. B.; Abraham, S.; Haley, G.; Kwan, B. P.; Hall, C.; Hough, G. W.; Shaw, K. J.; Finn, J., Identification of Bacteria-Selective Threonyl-tRNA Synthetase Substrate Inhibitors by Structure-Based Design. *J. Med. Chem.* **2013**, *56* (4), 1748-1760.
34. Hernandez, V.; Crepin, T.; Palencia, A.; Cusack, S.; Akama, T.; Baker, S. J.; Bu, W.; Feng, L.; Freund, Y. R.; Liu, L.; Meewan, M.; Mohan, M.; Mao, W.; Rock, F. L.; Sexton, H.; Sheoran, A.; Zhang, Y.; Zhang, Y. K.; Zhou, Y.; Nieman, J. A.; Anugula, M. R.; Keramane el, M.; Savariraj, K.; Reddy, D. S.; Sharma, R.; Subedi, R.; Singh, R.; O'Leary, A.; Simon, N. L.; De Marsh, P. L.; Mushtaq, S.; Warner, M.; Livermore, D. M.; Alley, M. R.; Plattner, J. J., Discovery of a Novel Class of Boron-based Antibacterials with Activity Against Gram-negative Bacteria. *Antimicrob. Agents Chemother.* **2013**, *57* (3), 1394-1403.



35. Artero, J. B.; Teixeira, S. C.; Mitchell, E. P.; Kron, M. A.; Forsyth, V. T.; Haertlein, M., Crystallization and Preliminary X-ray Diffraction Analysis of Human Cytosolic Seryl-tRNA Synthetase. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **2010**, *66* (Pt 11), 1521-1524.
36. Battye, T. G.; Kontogiannis, L.; Johnson, O.; Powell, H. R.; Leslie, A. G., iMOSFLM: a New Graphical Interface for Diffraction-image Processing with MOSFLM. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67* (Pt 4), 271-281.
37. Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S., Overview of the CCP4 Suite and Current Developments. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67* (Pt 4), 235-242.
38. Vonrhein, C.; Flensburg, C.; Keller, P.; Sharff, A.; Smart, O.; Paciorek, W.; Womack, T.; Bricogne, G., Data Processing and Analysis with the autoPROC Toolbox. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67* (Pt 4), 293-302.
39. McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J., Phaser crystallographic software. *Journal of Applied Crystallography* **2007**, *40* (4), 658-674.
40. Adams, P. D.; Afonine, P. V.; Bunkoczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H., PHENIX: a Comprehensive Python-based System for Macromolecular Structure Solution. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, *66* (Pt 2), 213-221.
41. Bricogne, G.; Blanc, E.; Brandl, M.; Flensburg, C.; Keller, P.; Paciorek, W.; Roversi, P.; Sharff, A.; Smart, O. S.; Vonrhein, C.; Womack, T. O., BUSTER version 2.10.3. Cambridge, United Kingdom: Global Phasing Ltd. **2017**.
42. Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K., Features and Development of Coot. *Acta Crystallographica Section D* **2010**, *66* (4), 486-501.
43. Brown, P. H.; Schuck, P., Macromolecular Size-and-Shape Distributions by Sedimentation Velocity Analytical Ultracentrifugation. *Biophys. J.* **2006**, *90* (12), 4651-4661.
44. Brautigam, C. A., Calculations and Publication-Quality Illustrations for Analytical Ultracentrifugation Data. *Methods Enzymol.* **2015**, *562*, 109-133.

## Table of contents graphic

